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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : G01N 33/48, C12Q 1/00 — A1

(11) International Publication Number: **WO 00/45165**

(43) International Publication Date: 3 August 2000 (03.08.00)

(21) International Application Number: PCT/US00/02329

(22) International Filing Date: 1 February 2000 (01.02.00)

(30) Priority Data:

60/118,102	1 February 1999 (01.02.99)	US
09/454,595	7 December 1999 (07.12.99)	US

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(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: METHODS OF IDENTIFYING THERAPEUTICALLY EFFECTIVE ANTINEOPLASTIC AGENTS WITH CULTURED CELLS HAVING INTACT CELL MEMBRANES AND CORRESPONDING PRODUCTS

(57) Abstract

A method for identifying potentially therapeutically effective antineoplastic compounds comprising determining the ability of test compounds to act as activators of the caspase cascade in viable cultured eukaryotic cells having an intact cell membrane and expressing a cancer phenotype, wherein a test compound that enhances caspase cascade activity is determined to have potential therapeutic efficacy. The method specifically differentiates activators of the caspase cascade from non–specific cell poisons. A therapeutic method useful to modulate *in vivo* apoptosis or *in vivo* neoplastic disease, comprising administering to a subject an effective amount of a compound identified as a caspase cascade activator is provided. Compounds, pharmaceutical compositions and a kit for performing the therapeutic method are further provided.

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The title does not conform to PCT rule 4.3 because it is too long. Text of a New Title: "METHODS OF IDENTIFYING THERAPEUTICALLY EFFECTIVE ANTINEOPLASTIC AGENTS WITH CULTURED CELLS HAVING INTACT CELL MEMBRANES AND CORRESPONDING PRODUCTS."

Background of the Invention

Field of the Invention

This invention relates to a method for identifying potential therapeutically effective antineoplastic agents. In particular, the invention relates to the use of viable cultured cells having an intact cell membrane to identify compounds that directly or indirectly activate the caspase cascade. Also taught is a method for using the potentially therapeutically effective antineoplastic agents and pharmaceutical compositions for their use.

Related Art

Cancer

Cancer is a group of diseases characterized by the uncontrolled growth and spread of abnormal cells. The occurrence of cancer increases as individuals age, and the majority of cases affect adults who are middle-aged or older. In the U.S., men have a 1 in 2 lifetime risk of developing cancer, whereas for women the risk is 1 in 3. More than 11 million new cancer cases have been diagnosed since 1990 in the U.S., and more than 1 million new cancer cases are diagnosed each year. Cancer is the second leading cause of death in the U.S., exceeded only by heart disease, and since 1990 there have been approximately 5 million cancer related deaths.

Antineoplastic Agents

The primary methods of treatment for cancer are surgery, irradiation, and chemotherapy with antineoplastic agents. Progress has been made in enhancing the effectiveness of each of these methods. Thus, curative treatments have been developed for such otherwise fatal cancers as leukemia and testicular cancer. It is equally true that there is still a major need for improvement in virtually every therapeutic method for the treatment of cancer, especially in the case of chemotherapy with antineoplastic agents (Hardman, Limbird, Molinoff, Ruddon and Gilman, Eds., *Goodman and Gilman's The Pharmacological Basis of Therapeutics,* Ninth Edition, McGraw-Hill, New York, pages 1225-1287 (1996)).

It is remarkable that the earliest antineoplastic agents were actually derived from the war gas "sulfur mustard" first used in World War I. The closely related "nitrogen mustards" were studied somewhat later. The first clinical studies using these compounds were undertaken in 1942, which began the modern use and development of antineoplastic agents. Even today, the nitrogen mustard drugs form a substantial part of the antineoplastic drug inventory. In view of the derivation of antineoplastic agents from war gases, it is not surprising that drug toxicity remains an important limitation of antineoplastic therapy.

An essential element in connection with the drug of a therapeutic agent is its therapeutic index. The therapeutic index is obtained by first determining the median effective dose, also termed the ED_{50}, which is the dose of the drug required to produce a specified effect in 50% of a test animal population. Next, the median lethal dose, or LD_{50}, is obtained by determining the dose that is lethal for 50% of an animal population. The ratio of the LD_{50} to the ED_{50} is the therapeutic index, or T.I. It is obviously desirable to use drugs with as high a T.I. value as possible, as these will be the safest drugs for any given purpose.

The T.I. values for the antineoplastic agents are typically among the lowest of any class of medicinal agents. This low T.I. is one of the greatest single problems facing the physician in treating a patient with a neoplastic disease. In addition to considering whether an antineoplastic agent is suited for treating the

disease afflicting the patient, the physician must consider the renal (kidney) and hepatic (liver) function of the patient, as well as the general state of health of the patient, and his or her willingness to undergo arduous or painful therapy. Thus, it is clear that much progress remains to be made in developing new, safer, and more effective antineoplastic agents, and more particularly, in methods for the identification of such agents.

An essential question to be answered in attempting to reduce the toxicity of an entire class of therapeutic agents, such as the antineoplastic agents, is the identity of the mechanisms by which the agents exert their therapeutic effect. The goal in this type of analysis is to obtain new agents that act by mechanisms producing fewer toxic effects.

The Cell Cycle

In the case of current antineoplastic drugs, the mechanism of action frequently involves an attack at specific phases of the cell cycle. In brief, the cell cycle refers to the stages through which cells normally progress during their lifetimes. Normally, cells exist in a resting phase termed G_o. During multiplication, cells progress to a stage in which DNA synthesis occurs, termed S. Later, cell division, or mitosis occurs, in a phase called M. Antineoplastic drugs such as cytosine arabinoside, hydroxyurea, 6-mercaptopurine, and methotrexate are S phase specific, whereas antineoplastic drugs such as vincristine, vinblastine, and paclitaxel are M phase specific. Many slow growing tumors, for example colon cancers, exist primarily in the G_o phase, whereas rapidly proliferating normal tissues, for example bone marrow, exist primarily in the S or M phase. Thus, a drug like 6-mercaptopurine can cause bone marrow toxicity while remaining ineffective for a slow growing tumor. Further aspects of the chemotherapy of neoplastic diseases are known to those skilled in the art (see, e.g., Hardman, Limbird, Molinoff, Ruddon and Gilman, Eds., *Goodman and Gilman's The Pharmacological Basis of Therapeutics,* Ninth Edition, McGraw-Hill, New York, pages 1225-1287 (1996)).

The Search for Antineoplastic Drugs

Because of the severity of the problem of neoplastic disease in a public health sense, as briefly indicated above, an extensive search for antineoplastic drugs has been undertaken in past years in government, academic, and industry laboratories. The National Cancer Institute has sponsored a wide-ranging empirical search for such agents for many years. Testing was undertaken in animals, usually mice, which were inoculated with tumor cell lines. The results of these empirical programs have been limited, but they provided the relatively unsatisfactory drugs that are in use today.

More recently, antimetabolite theory and rational drug design approaches have been used. The Nobel Prize in Medicine or Physiology was awarded to Gertrude Elion and George Hitchings for their extensive studies leading to new antineoplastic antimetabolites. Lengthy x-ray crystallographic studies on thymidilate synthase have led to inhibitors of the enzyme that are useful as antineoplastic agents. Nevertheless, none of these approaches, from the early "brute force" methods to the later elegant crystallographic work has resulted in "breakthrough" antineoplastic drugs.

Today, high throughput screening (HTS) and combinatorial chemistry offer the possibility of screening thousands of compounds, even hundreds of thousands of compounds, in a relatively short time. The availability of automated methods has been an important factor in this development. These methods obviously cannot be applied to whole animal screening, but must be restricted to cellular or macromolecular targets. Thus, a long felt need exists for assay methods suitable for the HTS discovery of effective new antineoplastic agents, which is uniquely solved by the present invention.

Prior cell based assays involved treatment of cell lines for prolonged periods with potential antineoplastic drugs. For example, the National Cancer Institute screen involves a panel of 60 cell lines that are treated for a prolonged period, for example 48-60 hours. The control cells are then counted and compared to the number of cell colonies in the treated samples. These methods,

which result in dead or dying cells, have been disappointing in that they have failed to identify drugs that are effective in the presence of tumor lines with mutated p53 phenotypes.

Apoptosis

A normal checkpoint in the life of cells in multicellular organisms is the process of apoptosis (see, e.g., Evan and Littlewood, *Science 281*:1317-1322 (1998). Apoptosis is the highly conserved mechanism by which cells commit suicide. Characteristics of the process include an execution phase that includes loss of cell volume, plasma membrane blebbing and chromatin condensation, followed by packing of the cellular contents into membrane-enclosed vesicles called apoptotic bodies that are rapidly phagocytosed by neighboring cells. Apoptosis differs from necrosis, which is cell death resulting from physical injury.

Apoptosis is one of several mechanisms that cells employ in response to the hazards raised by DNA damage. In particular, apoptosis is an effective action for damaged cells that can replicate and become cancerous. One major mediator of apoptosis is p53, which is a transcription factor that is normally present in low amounts because it is subject to a destruction process signaled by the Mdm-2 protein. Additionally, p53 can induce growth arrest in cells. In human cancer, p53 is often functionally inactivated so that its growth arrest activity or apoptotic activity is diminished. Thus, a therapeutic treatment for cancer, in which normal apoptosis is diminished, would be to enhance the apoptotic process through the administration of appropriate drugs. Moreover, since autoimmune disease and certain degenerative diseases also involve the proliferation of abnormal cells, therapeutic treatment for these diseases could also involve the enhancement of the apoptotic process through the administration of appropriate drugs.

It is pertinent, therefore, to inquire into the mechanism of apoptosis in order to develop a method for the identification of such antineoplastic and related drugs. It has been found that a group of proteases are a key element in apoptosis

(see, e.g. Thornberry, *Chemistry and Biology* 5:R97-R103 (1998); Thornberry, *British Med. Bull. 53*:478-490 (1996)). Genetic studies in the nematode *Caenorhabditis elegans* revealed that apoptotic cell death involves at least 14 genes, two of which are the pro-apoptotic (death-promoting) *ced* (for *cell death abnormal*) genes, *ced-3* and *ced-4*. CED-3 is homologous to interleukin 1β-converting enzyme, a cysteine protease, which is now called caspase-1. When these data were ultimately applied to mammals, and upon further extensive investigation, it was found that the mammalian apoptosis system appears to involve a cascade of caspases, or a system that behaves like a cascade of caspases. At present, the caspase family of cysteine proteases comprises 10 different members, and more may be discovered in the future. All known caspases are synthesized as zymogens that require cleavage at an aspartyl residue prior to forming the active enzyme. Thus, caspases are capable of activating other caspases, in the manner of an amplifying cascade.

The caspase cascade can be involved in disease processes in two major aspects. Excessive activity of the caspase cascade can lead to excessive apoptosis and organ failure. Among the diseases that could result from this excessive activity are myocardial infarction, congestive heart failure, viral infections, rheumatoid arthritis and others. Inhibitors of the caspase cascade could thus be candidates for therapeutic intervention in such diseases. Inasmuch as methods for the discovery of enzyme inhibitors is a frequently practiced art, numerous approaches to the discovery of caspase inhibitors are available (see Villa *et al.*, *Trends Biochem Sci. 22*:388-393, (1997); Liang and Fesik, *J. Mol. Biol. 274*:291-302 (1997)).

Caspase Cascade Activators

Although the development of enzyme inhibitors as therapeutic agents is a well understood art (see Muscate and Kenyon, *Burger's Medicinal Chemistry 1*:733-782, 5th Ed. (1995)) this is not the case in the development of enzyme activators. The theoretical basis for the development of enzyme activators is still

in its infancy. In the case of the apoptosis process, control points are known to exist that represent points for intervention leading to activation. These control points include the CED-9—BCL-like and CED-3—ICE-like gene family products, which are intrinsic proteins regulating the decision of a cell to survive or die and executing part of the cell death process itself, respectively (see Schmitt *et al., Biochem. Cell. Biol., 75*:301-314 (1997)). BCL-like proteins include BCL-xL and BAX-α, which appear to function upstream of caspase activation. BCL-xL appears to prevent activation of the apoptotic protease cascade, whereas BAX-α accelerates activation of the apoptotic protease cascade. Thus, it is clear that the possibility exists for the activation of the caspase cascade, although the exact mechanisms for doing so are not clear at this point. Inasmuch as it is equally clear that insufficient activity of the caspase cascade and consequent apoptotic events are implicated in various types of cancer and possibly in various degenerative and autoimmune diseases, the development of caspase cascade activators is a highly desirable goal in the development of potentially therapeutically effective antineoplastic agents.

In order to identify caspase cascade activators that are potentially therapeutically effective antineoplastic agents, a number of issues must be resolved that have not been adequately addressed by the prior art. First, inasmuch as about half of all cancers have mutations of p53, it is important to use a method that activates the caspase cascade directly or indirectly, independently of p53. This means that an isolated enzyme assay, wherein the enzyme is an individually isolated caspase, or a caspase assay in dead cells (see Los *et al., Blood*, 90:3118-3129, (1997)) is unsuitable because receptors and/or cofactors that influence the caspase cascade might not be present in such preparations. In addition, because the caspase cascade is intracellular, compounds that influence it must ordinarily cross the cell membrane, in contrast to many other types of drugs that interact with the external elements of transmembrane receptors. Thus, an assay in viable cells having an intact cell membrane must be used for the present purpose. Thirdly, any assay for this purpose should be amenable to a

high-throughput mode embodiment if it is to be useful for broadly based drug screening. Thus, it should be an assay that can be carried out quickly.

However, there are substantial technical obstacles to the development of cellular assays for caspase activators. Prior attempts to make the discovery of potentially therapeutically effective antineoplastic agents that act as caspase activators more accurate, cost effective, and rapid, have not achieved the simplicity and reliability of operation of the present invention.

Identification of compounds that modulate caspase cascade activity, such as those that act as specific activators, may not only aid in elucidating the function of caspases, but may yield therapeutically useful compounds. In particular, compounds that specifically activate specific elements of the caspase cascade should be useful in identifying essential characteristics of those elements and should aid in the design of therapeutic disease specific agents.

Therefore, the teachings of the present invention provide a method for identifying compounds that activate the caspase cascade independent of the p53 transcription factor in viable cultured eukaryotic cells having an intact cell membrane. The present invention is also directed to the use of compounds that act as activators of the caspase cascade in viable cultured eukaryotic cells having an intact cell membrane. Such compounds should be useful as therapeutic agents for the treatment of caspase cascade-mediated diseases and disorders, such as neoplastic diseases and disorders, and also for the identification of the function of the caspase cascade.

WO98/55863 discloses a microscale method of determining the specific apoptotic activity of a cell. According to this application, an accessory reagent is required. Such accessory reagents include reagents that allow for the lysis, solubilization or permeabilization of the cell membrane.

These and other objects are accomplished by the teachings of the present invention, the nature of which is set forth in the following description and distinctly pointed out and set forth in the appended claims.

Summary of the Invention

The present invention is directed to methods for identifying direct and indirect activators of the caspase cascade, therapeutic methods to use such activators, compositions for such activators, and kits comprising such activators.

More particularly, the invention relates to a method for identifying potentially therapeutically effective antineoplastic compounds by determining the ability of test compounds to activate the caspase cascade in viable cultured eukaryotic cells having an intact cell membrane when the cells are exposed to test compounds for a predetermined period of time at a predetermined temperature, wherein a test compound is determined to have potential therapeutic efficacy if said caspase cascade activity is enhanced in response to the presence of said test compound.

In particular, the invention relates to a method for identifying a potentially therapeutically effective antineoplastic compound comprising determining the ability of a test compound to act as an activator of the caspase cascade in viable cultured eukaryotic cells having an intact cell membrane, wherein a test compound is determined to have potential therapeutic efficacy if said caspase cascade activity is enhanced in response to the presence of said test compound, said method comprising:

obtaining viable cultured eukaryotic cells having an intact cell membrane expressing a cancer phenotype by culturing in a cell growth medium under conditions which result in growth;

exposing said viable cultured cells to the test compound for a predetermined period of time at a predetermined temperature;

adding a reporter compound having at least one measurable property which is responsive to the caspase cascade;

measuring the caspase cascade activity of said exposed viable cultured cells by measuring said at least one measurable property; and,

wherein an increase in the measured caspase cascade activity in the presence of said test compound is an indication that said test compound is a potential therapeutically effective antineoplastic compound.

A permeabilization enhancer or agent may be added in combination with the test compound. Preferably, a reporter compound for measuring caspase activity with or without a permeabilization enhancer is added to the cell after the test compound has been incubated with the cell for a predetermined time to allow transport of the test compound across the cell membrane or interaction of the test compound with receptors on the surface of the cell membrane. The predetermined period of time may be about 1 minute to less than about 24 hours, preferably about 1-24 hours, and most preferably about 3, 5, or 24 hours. The predetermined temperature may be about 4 °C to about 50 °C, preferably about 37 °C. In contrast to previously used methods, which employ time periods of 48-72 hours, the shorter predetermined period of time used in the present invention is sufficiently short to maintain an intact cell membrane in the cells being used in the assay, and makes possible the specificity of the present method for activators of the caspase cascade that are potentially therapeutically effective antineoplastic agents, rather than nonspecific cell poisons, etc. The intactness of the cell membrane may be confirmed by use of propidium iodide (available from Alrich Chemical Co.) as set forth in Example 1. In contrast to methods previously known in the art, wherein non-viable cells were used to measure apoptosis, the method of the present invention detects therapeutically important caspase cascade activator drugs, ignores non-specific cell poisons, differentiates among known antineoplastic agents such as p53-dependent agents vs. p53 independent agents, and rapid caspase activator agents vs. other antineoplastic agents. Thus, in contrast to currently available methods, the method of the present invention offers important advantages regarding therapeutic relevance and selectivity in the discovery of new antineoplastic agents.

The invention also relates to methods for assaying the potency of a potentially therapeutically effective antineoplastic compound as an activator of the caspase cascade in viable cultured eukaryotic cells having an intact cell membrane.

The invention also relates to a method for assaying the potency of a potential therapeutically effective antineoplastic compound that functions as an activator of the caspase cascade in viable cultured eukaryotic cells having an intact cell membrane, said method comprising:

obtaining a first and a second population of viable cultured eukaryotic cells having an intact cell membrane expressing a cancer phenotype by culturing said eukaryotic cells in a cell growth medium under conditions which result in growth;

exposing said first population to a predetermined amount of a test compound dissolved in a solvent for a predetermined period of time at a predetermined temperature;

exposing said second population to an amount of the solvent which was used to dissolve said test compound for said predetermined period of time at said predetermined temperature;

adding to said test compound-exposed first population and said solvent-exposed second population a reporter compound having at least one measurable property which is responsive to the caspase cascade;

measuring said at least one measurable property of said reporter compound in said test compound-exposed first population and thereby measuring the caspase cascade activity of said test compound-exposed first population;

measuring said at least one measurable property of said reporter compound in said solvent-exposed second population and thereby measuring the caspase cascade activity of said solvent-exposed second population; and

calculating the ratio of caspase cascade activity measured for said test compound-exposed first population compared to said caspase cascade activity measured for said solvent-exposed second population to determine the relative potency of the test compound as an activator of the caspase cascade.

The invention also relates to a method for assaying the potency of a test compound to synergise with other cancer chemotherapeutic agents as an activator of the caspase cascade. This method may also be employed with eukaryotic cells including, but not limited to, p53 deficient cells, Bcl-2 overexpressing family member cells, ataxia telengiectasia mutated cells, surviving overexpressing cells, bcr/abl mutated cells, p16 mutated cells, Brca1 mutated cells, Brca2 mutated cells, multi-drug resistance cells, and DNA mismatch repair deficiency cells.

This aspect of the invention relates to a method for assaying the potency of a test compound to synergise with a known cancer chemotherapeutic agent by functioning as an activator of the caspase cascade, said method comprising:

obtaining a first and a second population of viable cultured eukaryotic cells having an intact cell membrane expressing a cancer phenotype by culturing in a cell growth medium under conditions which result in growth;

exposing said first population to a combination of a predetermined amount of a test compound and a subinducing amount of a known cancer chemotherapeutic agent for a first predetermined period of time, at a first predetermined temperature;

exposing said second population to an amount of solvent which was used to dissolve the test compound and to a subinducing amount of a known cancer chemotherapeutic agent for said first predetermined period of time at said first predetermined temperature;

adding a reporter compound to said exposed first population and to said exposed second population, said reporter compound having at least one measurable property which is responsive to the caspase cascade;

incubating the resulting mixture of said first population, said test compound, said known cancer chemotherapeutic agent and said reporter compound for a second predetermined time period at a second predetermined temperature;

incubating the resulting mixture of said second population, said solvent, said known chemotherapeutic agent and said reporter compound for said second predetermined time period at said second predetermined temperature;

measuring said at least one measurable property of said reporter compound in said first resulting mixture and thereby measuring the caspase cascade activity of said first population in said first resulting mixture;

measuring said at least one measurable property of said reporter compound in said second resulting mixture and thereby measuring the caspase cascade activity of said second population in said second resulting mixture; and,

calculating the ratio of measured caspase cascade activities of said first population to said second population to determine whether said test compound acts synergistically with said known cancer chemotherapeutic agent as an activator of the caspase cascade.

Also provided is a method for carrying out any of the forgoing methods in an automated apparatus such as Zymark Allegro™ (Zymark, Inc. Hopkinton, MA) or Beckman Multimek™ (Beckman Instruments, Fullerton, CA). In a preferred embodiment, the method is carried out in an automated measurement apparatus; said apparatus comprising:

a controller (e.g. a computer interface control) for coordinating the operation of the apparatus;

sample-containing plate comprising a plurality of wells;

moving equipment, controlled by said controller for moving at least one said well to a predetermined position;

liquid transfer equipment controlled by said controller for adding predetermined amounts of at least one liquid to at least one said well in a predetermined position; environmental control equipment controlled by said controller for controlling the environment (e.g. temperature or atmosphere) of said sample-containing means in a predetermined position; and

measurement equipment controlled by said controller for measuring at least one attribute (e.g. fluorescence) in at least one said well in said sample-containing plate.

The present invention includes a therapeutic method useful to modulate *in vivo* apoptosis or *in vivo* neoplastic disease, comprising administering, to an animal in need of such treatment, an effective amount of a compound, preferably

with a measured ratio of at least 2, and most preferably with a measured ratio greater than a statistically significant value calculated as (Ave Control RFU + 4 x $SD_{Control}$) / (Ave Control RFU) for that run, or a pharmaceutically acceptable salt or prodrug of the compound, which functions as a caspase cascade activator in the method of the invention, provided that the compound is not a known apoptosis modulator and/or antineoplastic agent prior to its identification as a caspase cascade activator in the method of the invention.

The present invention also includes a kit for performing this therapeutic method, comprising packaging material containing the compound, or a pharmaceutically acceptable salt of the compound, and including a package label indicating that the compound, or a pharmaceutically acceptable salt or prodrug of the compound, is useful to modulate *in vivo* apoptosis, or one or more *in vivo* neoplastic diseases.

In practicing the therapeutic methods, effective amounts of compositions containing therapeutically effective concentrations of the compounds formulated for oral, intravenous, local or topical application, for the treatment of neoplastic diseases and other diseases in which caspase cascade mediated physiological responses are implicated, are administered to an animal exhibiting the symptoms of one or more of these disorders. The amounts are effective to ameliorate or eliminate one or more symptoms of the disorders.

In another embodiment, a pharmaceutical composition comprising a compound, or a pharmaceutically acceptable salt of said compound, which functions as a caspase cascade activator according to the assay method of the invention, preferably where the ratio is at least 2, and most preferably with a measured ratio greater than a statistically significant value calculated as (Ave Control RFU + 4 x $SD_{Control}$) / (Ave Control RFU) for that run, in combination with a pharmaceutically acceptable vehicle is provided.

Another embodiment of the present invention is directed to a composition effective to inhibit neoplasia comprising a first compound, or a pharmaceutically acceptable salt or prodrug of said compound, which functions as a caspase cascade activator according to the assay method of the invention, preferably

wherein the ratio is at least about 2, in combination with a second compound, or a pharmaceutically acceptable salt of said compound, which is a known cancer chemotherapeutic agent. A composition, in which the inhibition achieved by a predetermined amount of the combination is greater than the additive effect achieved by using the first compound and the second compound individually in the amounts that each comprises in the predetermined amount of the composition is also taught.

Brief Description of the Drawing

Fig. 1 depicts a bar graph showing the ratio calculated for the compounds listed in Table 2 as well as the standard deviation.

Detailed Description of the Preferred Embodiments

Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of ordinary skill in the art to which this invention belongs.

As used herein, apoptosis is a highly conserved, genetically programmed form of cellular suicide characterized by distinct morphological changes such as cytoskeletal disruption, cell shrinkage, membrane blebbing, nuclear condensation, fragmentation of DNA, and loss of mitochondrial function.

As used herein, a caspase is a cysteine protease of the interleukin-1β/CED-3 family. As used herein, the caspase cascade is a sequential activation of at least two caspases, or the activation of caspase activity that behaves as if it involves the sequential activation of at least two caspases.

As used herein, cancer is a group of diseases characterized by the uncontrolled growth and spread of abnormal cells or one in which compounds that activate the caspase cascade have therapeutic use. Such diseases include, but

are not limited to, Hodgkin's disease, non-Hodgkin's lymphomas, acute and chronic lymphocytic leukemias, multiple myeloma, neuroblastoma, breast carcinomas, ovarian carcinomas, lung carcinomas, Wilms' tumor, cervical carcinomas, testicular carcinomas, soft-tissue sarcomas, chronic lymphocytic leukemia, primary macroglobulinemia, bladder carcinomas, chronic granulocytic leukemia, primary brain carcinomas, malignant melanoma, small-cell lung carcinomas, stomach carcinomas, colon carcinomas, malignant pancreatic insulinoma, malignant carcinoid carcinomas, malignant melanomas, choriocarcinomas, mycosis fungoides, head and neck carcinomas, osteogenic sarcoma, pancreatic carcinomas, acute granulocytic leukemia, hairy cell leukemia, neuroblastoma, rhabdomyosarcoma, Kaposi's sarcoma, genitourinary carcinomas, thyroid carcinomas, esophageal carcinomas, malignant hypercalcemia, cervical carcinomas, renal cell carcinomas, endometrial carcinomas, polycythemia vera, essential thrombocytosis, adrenal cortex carcinomas, skin cancer, and prostatic carcinomas.

As used herein an effective amount of a compound for treating a particular disease is an amount that is sufficient to ameliorate, or in some manner reduce, the symptoms associated with the disease. Such amount may be administered as a single dosage or may be administered according to a regimen, whereby it is effective. The amount may cure the disease but, typically, is administered in order to ameliorate the disease. Typically, repeated administration is required to achieve the desired amelioration of symptoms.

As used herein, a subinducing amount of a substance is an amount that is sufficient to produce a measurable change in caspase cascade activity when used in the method of the present invention and which produces a greater measurable change in caspase cascade activity when used in synergistic combination with a test compound in the method of the present invention.

Examples of known anti-cancer compounds which can be used in the practice of the invention, e.g. to screen for compounds with synergistic apoptotic induction activity and for combination therapy include, but are not limited to alkylating agents such as busulfan, cis-platinum, mitomycin C, and carboplatin;

antimitotic agents such as colchicine, vinblastine, paclitaxel, and docetaxel; topo I inhibitors such as camptothecin and topotecan; topo II inhibitors such as doxorubicin and etoposide; RNA/DNA antimetabolites such as 5-azacytidine, 5-fluorouracil and methotrexate; and DNA antimetabolites such as 5-fluoro-2'-deoxyuridine, ara-C, hydroxyurea and thioguanine.

As used herein, an activator of the caspase cascade is a compound, such as a drug or antibody that enhances caspase-mediated physiological responses such as cellular apoptosis. The activator may act by any one or a combination of mechanisms.

As used herein, the biological activity of the caspase cascade includes any activity induced, potentiated or influenced by the caspase cascade *in vivo*.

As used herein, EC_{50} refers to a dosage, concentration or amount of a particular test compound that elicits a dose-dependent response at 50% of maximal expression of a particular response that is induced, provoked or potentiated by the particular test compound.

As used herein, pharmaceutically acceptable salts or prodrugs of the compounds include any salts, esters or derivatives that may be readily prepared by those of skill in this art using known methods for such derivatization and that produce compounds that may be administered to animals or humans without substantial toxic effects and that either are pharmaceutically active or are prodrugs. For example, hydroxyl groups can be esterified (*e.g.*, with a C_{2-10} alkanoyl group or succinic acid) or etherified (with a C_{1-6} alkoxy methylchloride). In addition, a carboxylic acid group maybe esterified (e.g. with a C_{1-6} alcohol).

As used herein, a prodrug is a compound that, upon *in vivo* administration, is metabolized or otherwise converted to the biologically, pharmaceutically or therapeutically active form of the compound. To produce a prodrug, the pharmaceutically active compound is modified such that the active compound will be regenerated by metabolic processes. The prodrug may be designed to alter the metabolic stability or the transport characteristics of a drug, to mask side effects or toxicity, to improve the flavor of a drug or to alter other characteristics or properties of a drug. By virtue of knowledge of

pharmacodynamic processes and drug metabolism *in vivo*, those of skill in this art, once a pharmaceutically active compound is known, can design prodrugs of the compound (see, e.g., Nogrady, *Medicinal Chemistry: A Biochemical Approach*, Oxford University Press, New York, pages 388-392 (1985)). For example, succinylsulfathiazole is a prodrug of 4-amino-*N*-(2-thiazoyl)benzenesulfonamide (sulfathiazole) that exhibits altered transport characteristics.

As used herein, treatment means any manner in which the symptoms of a condition, disorder or disease are ameliorated or otherwise beneficially altered.

As used herein, amelioration of the symptoms of a particular disorder by administration of a particular pharmaceutical composition refers to any lessening, whether permanent or temporary, lasting or transient, that can be attributed to or associated with administration of the composition.

As used herein, substantially pure means sufficiently homogeneous to appear free of readily detectable impurities as determined by standard methods of analysis, such as thin layer chromatography (TLC), gel electrophoresis and high performance liquid chromatography (HPLC), used by those of skill in the art to assess such purity, or sufficiently pure such that further purification would not detectably alter the physical and chemical properties, such as enzymatic and biological activities, of the substance. Methods for purification of the compounds to produce substantially chemically pure compounds are known to those of skill in the art. A substantially chemically pure compound, however, may be a mixture of stereoisomers. In such instances, further purification might increase the specific activity of the compound.

As used herein, biological activity refers to the *in vivo* activities of a compound or physiological responses that result upon *in vivo* administration of a compound, composition or other mixture. Biological activity, thus, encompasses therapeutic effects and pharmaceutical activity of such compounds, compositions, and mixtures.

As used herein, a fluorogenic, chromogenic or chemiluminescent substrate is a substance that produces fluorescence, light absorption within the

ultraviolet, visible or infrared spectrum, or light emission under the influence of the caspase cascade. Example of substrates which are useful for the screening including *N*-(Ac-DEVD)-*N'*-acetyl-Rhodamine 110 (SEQ ID NO:1), *N*-(Ac-DEVD)-*N'*-ethoxycarbonyl-Rhodamine 110 (SEQ ID NO:1), *N*-(Ac-DEVD)-*N'*-hexyloxycarbonyl-Rhodamine 110 (SEQ ID NO:1), *N*-(Ac-DEVD)-*N'*-octyloxycarbonyl-Rhodamine 110 (SEQ ID NO:1), *N*-(Ac-DEVD)-*N'*-decyloxycarbonyl-Rhodamine 110 (SEQ ID NO:1), *N*-(Ac-DEVD)-*N'*-dodecyloxycarbonyl-Rhodamine 110 (SEQ ID NO:1), *N*-(Ac-DEVD)-*N'*-2-butoxyethoxycarbonyl-Rhodamine 110 (SEQ ID NO:1), *N*-(Ac-DEVD)-*N'*-(ethylthio)carbonyl-Rhodamine 110 (SEQ ID NO:1), *N*-(Ac-DEVD)-*N'*-(hexylthio)carbonyl-Rhodamine 110 (SEQ ID NO:1), *N*-(Ac-DEVD)-*N'*-(octylthio)carbonyl-Rhodamine 110 (SEQ ID NO:1), *N*-(Ac-DEVD)-*N'*-(*N*-hexyl-*N*-methylcarbamyl)-Rhodamine 110 (SEQ ID NO:1), *N*-(Ac-DEVD)-*N'*-(2,3,4,5,6-pentafluorobenzoyl)-Rhodamine (SEQ ID NO:1), *N*-(Ac-DEVD)-*N'*-(2,3,4,5-tetrafluorobenzoyl)-Rhodamine (SEQ ID NO:1) and others disclosed in pending U.S. patent application Serial No. 09/168,888, filed October 9, 1998, and international patent application No. PCT/US98/21231, filed October 9, 1998 which applications are herein incorporated by reference in their entirety. Since they are relatively small in size and lipophilic at the same time, many of these substrates can be used in the assays of the invention in the absence of a permeabilization enhancer.

Such substrates may be prepared by a process comprising condensation of Rhodamine 110 with an acylating agent (e.g. acetic anhydride or ethylchloroformate) to give the mono-acylated Rhodamine 110 (e.g. Rhodamine 110 mono-acetate), followed by condensation with N-Ac-Asp(OBu-t)-Glu(OBu-t)-Val-Asp(OBu-t) (SEQ ID NO:1) with EDC/pyr/DMF, and removal of the t-butyl groups with trifluoroacetic acid.

Other useful substrate includes Ac-DEVD-*p*NA (SEQ ID NO:1), Ac-DEVD-AMC (SEQ ID NO:1), MCA-DEVDAPK(DNP)-OH (SEQ ID NO:2), Z-DEVD-AFC (SEQ ID NO:1), MCA-VDQMDGW[K-DNP]-NH_2 (SEQ ID NO:3), MCA-DEVDAR[K-DNP]-NH_2 (SEQ ID NO:4), Z-VDVAD-AFC (SEQ ID

NO:5), MCA-VDVADGW[K-DNP]-NH_2 (SEQ ID NO:6), MCA-VDQVDGW[K-DNP]-NH_2 (SEQ ID NO:7), Ac-VEID-*p*NA (SEQ ID NO:8), Ac-VEID-AMC (SEQ ID NO:8), Z-VEID-AFC (SEQ ID NO:8) and MCA-VQVDGW[K-DNP]-NH_2 (SEQ ID NO:9), (CALBIOCHEM, California).

As used herein, the abbreviations for any protective groups, amino acids, and other compounds, are, unless indicated otherwise, in accord with their common usage, or recognized abbreviations.

A. Identifying Compounds That Activate the Caspase Cascade

In one aspect, the invention relates to a method for identifying potentially therapeutically effective antineoplastic compounds by determining the ability of test compounds to activate the caspase cascade in viable cultured eukaryotic cells having an intact cell membrane, wherein a test compound is determined to have potential therapeutic efficacy if said caspase cascade activity is enhanced in response to the presence of said test compound, the method comprising (a) obtaining viable cultured eukaryotic cells having an intact cell membrane expressing a cancer phenotypé by culturing those cells in a cell growth medium under conditions which result in growth; (b) exposing the viable cultured cells to a test compound for a predetermined period of time at a predetermined temperature; (c) adding a reporter compound having at least one measurable property which is responsive to the caspase cascade; (d) measuring the caspase cascade activity of said exposed viable cultured cells by measuring said at least one measurable property of said reporter compound; and (e) wherein an increase in the measured caspase cascade activity in the presence of the test compound is an indication that the test compound is a potentially therapeutically effective antineoplastic compound.

In another aspect, the invention relates to a method for assaying the potency of a potentially therapeutically effective antineoplastic compound that functions as an activator of the caspase cascade in viable cultured eukaryotic cells having an intact cell membrane, comprising:

(a) obtaining a first and a second population of viable cultured eukaryotic cells, each of which having an intact cell membrane expressing a cancer phenotype, by culturing said eukaryotic cells in a cell growth medium under conditions which result in growth;

(b) exposing the first population to a predetermined amount of a test compound for a predetermined period of time at a predetermined temperature;

(c) exposing the second population to an amount of solvent that was used to dissolve the test compound for the predetermined period of time at the predetermined temperature;

(d) adding to said test compound-exposed first population and said solvent-exposed second population a reporter compound having at least one measurable property which is responsive to the caspase cascade;

(e) measuring said at least one measurable property of said reporter compound in said test compound-exposed first population and thereby measuring the caspase cascade activity of the test compound-exposed first population;

(f) measuring said at least one measurable property of said reporter compound in said solvent-exposed second population and thereby measuring the caspase cascade activity of the solvent-exposed second population; and

(g) calculating the ratio of caspase cascade activity measured for the test compound-exposed first population of cells to the caspase cascade activity measured for the solvent-exposed second population of cells to determine the relative potency of the test compound as an activator of the caspase cascade.

The caspase cascade activity measured for test compounds by this method can be compared to that measured for compounds which are known to affect enzymes involved in the apoptosis cascade to generate a measure of the relative effectiveness of the test substance. Compounds that can be used in comparison include known activators of enzymes involved in the apoptosis cascade. Known activators, either by direct or indirect mechanisms, of enzymes involved in the apoptosis cascade include but are not limited to vinblastine, etoposide (Yoon, H.J., *et al.*, *Biochim. Biophys. Acta. 1395*:110-120 (1998)) and doxorubicin (Gamen, S., *et al.*, *FEBS Lett. 417*:360-364 (1997)) which are topoisomerase II

inhibitors; cisplatin (Maldonado *et al.*, *Mutat. Res. 381*:67-75 (1997)); chlorambucil (Hickman, J.A., *Cancer Metastasis Rev. 11*:121-139 (1992)) which is an alkylating agent; and fluorouracil, an RNA/DNA anti-metabolite (Hickman, J.A., *Cancer Metastasis Rev. 11*:121-139 (1992)).

In a preferred embodiment, a plurality of viable cultured cells are exposed separately to a plurality of test compounds, e.g. in separate wells of a microtiter plate. In this embodiment, a large number of test compounds may be screened at the same time.

In another aspect, the invention relates to a method for assaying the potency of a test compound to synergise with other cancer chemotherapeutic agents as an activator of the caspase cascade, comprising (a) obtaining a first and a second population of viable cultured eukaryotic cells, having an intact cell membrane expressing a cancer phenotype, by culturing the cell populations in a cell growth medium under conditions which result in growth; (b) exposing the first population to a combination of a predetermined amount of a test compound and a subinducing amount of a known cancer chemotherapeutic agent for a first predetermined period of time at a first predetermined temperature; (c) exposing the second population to an equal amount of solvent, which was used to dissolve the test compound, and a subinducing amount of a known cancer chemotherapeutic agent for said first predetermined period of time at said first predetermined temperature; (d) adding a reporter compound to the exposed first population and to the exposed second population, the reporter compound having at least one measurable property which is responsive to the caspase cascade; (e) incubating the resulting mixture of the first population, the test compound, the known cancer chemotherapeutic agent and the reporter compound for a second predetermined time period at a second predetermined temperature; (f) incubating the resulting mixture of said second population, said solvent, said known chemotherapeutic agent, and said reporter compound for a second predetermined time period at a second predetermined temperature; (g) measuring said at least one measurable property of said reporter compound in said first resulting mixture and thereby measuring the caspase cascade activity of the first population in the

first resulting mixture; (h) measuring said at least one measurable property of the reporter compound in the second resulting mixture and thereby measuring the caspase cascade activity of the second population in the second resulting mixture; and (i) calculating the ratio of the caspase cascade activity of the first resulting mixture to the caspase cascade activity of the second resulting mixture to determine whether said test compound acts synergistically with the known cancer chemotherapeutic agent.

Reporter compounds are preferably used as a means for measuring caspase cascade activity in the whole-cell assays of the present invention.

Typical reporter compounds include fluorogenic, chromogenic or chemiluminescent compounds applied to cells or tissues containing cells at a concentration of about 0.01 nanomolar to about 0.1 molar, or an equivalent amount of a salt or prodrug thereof. A most preferred concentration is about 10 micromolar.

The test compounds are preferably presented to the cells or cell lines dissolved in a solvent. Preferred solvents include, DMSO, water and/or buffers. A most preferred solvent is DMSO. The preferred amount of DMSO is generally below 2% and most preferably 1% or below. At this concentration, DMSO functions as a solubilizer for the test compounds and not as a permeabilization agent. The amount of solvent tolerated by the cells must be checked initially by measuring cell viability or caspase induction with the different amounts of solvent alone to ensure that the amount of solvent has no effect on the cellular properties being measured.

Suitable buffers include cellular growth media, for example Iscove's media (Life Technologies, Inc.) with or without 10% fetal bovine serum. Other known cellular incubation buffers include phosphate, PIPES or HEPES buffers. One of ordinary skill in the art can identify other suitable buffers with no more than routine experimentation.

The cells can be derived from any organ or organ system for which it is desirable to find a potentially therapeutically effective antineoplastic compound that functions as an activator of the caspase cascade in viable cultured eukaryotic

cells having an intact cell membrane. Cellular genotypes for screening of test compounds include, but are not limited to, cells that are P53 negative, Bcl-2 overexpressing, Bcl-xL overexpressing, ataxia telengiectasia mutated (e.g. ATCC CRL 7201), multi-drug resistance (e.g. P-glycoprotein overexpressing, ATCC CRL-1977), DNA mismatch repair deficiency (e.g., defects in hMSH2, hMSH3, hMSH6, hPMS2, or hPMS1), HL-60 cells (ATCC CCL-240), SH-SY5Y cells (ATCC CRL-2266), and Jurkat cells (ATCC TIB-152), surviving overexpressing (e.g. ATCC CCL-185), bcr/abl mutated (eg ATCC CCL-243), p16 mutated, Brca1 mutated (e.g. ATCC CRL-2336), or Brca2 mutated. These and other cells may be obtained from the American Type Culture Collection, Manassas, VA.

Suitable solubilizers may be used for presenting reporter compounds to cells or cell lines. Preferred solubilizers include aqueous solutions of the test compounds in water-soluble form, for example as water-soluble salts. More preferably, the test compounds are dissolved in a buffer solution containing 20% sucrose (Sigma) 20 mM DTT (Sigma), 200 mM NaCl (Sigma), and 40 mM Na PIPES buffer pH 7.2 (Sigma).

Inasmuch as the caspase cascade takes place in the intracellular environment, measures may be undertaken to enhance transfer of the reporter compound across the cell membrane. This can be accomplished with a suitable permeabilization agent. Preferable permeabilization agents include, but are not limited to, NP-40, n-octyl-O-D-glucopyranoside, n-octyl-O-D-thioglucopyranoside, taurocholic acid, digitonin, CHAPS, lysolecithin, dimethyldecylphosphine oxide (APO-10), dimethyldodecylphosphine oxide (APO-12), N,N-bis-(3-D-gluconamidopropyl)cholamide (Big Chap), N,N-bis-(3-D-gluconamidopropyl)deoxycholamide (Big Chap, deoxy), BRIG-35, hexaethyleneglycol (C10E6), C10E8, C12E6, C12E8, C12E9, cyclohexyl-n-ethyl-O-D-maltoside, cyclohexyl-n-hexyl-O-D-maltoside, cyclohexyl-n-methyl-O-D-maltoside, polyethylene glycol lauryl ether (Genapol C-100), polyethylene glycol dodecyl ether (Genapol X-80), polyoxyethylene isotridecyl ether (Genapol X-100), n-decanoylsucrose, n-decyl-O-D-glucopyranoside, n-decyl-O-D-maltopyranoside, n-decyl-O-D-thiomaltoside, n-dodecanoylsucrose,

n-dodecyl-O-D-glucopyranoside, n-dodecyl-O-D-maltoside, n-heptyl-O-D-glucopyranoside, n-heptyl-O-D-thioglucopyranoside, n-hexyl-O-D-glucopyranoside, n-nonyl-O-D-glucopyranoside, n-octanoylsucrose, n-octyl-O-D-maltopyranoside, n-undecyl-O-D-maltoside, n-octanoyl-O-D-glucosylamine (NOGA), PLURONIC® F-127, and PLURONIC® F-68.

The cell lines are exposed to a predetermined amount of test compounds at concentrations in the range from about 1 picomolar to about 1 millimolar, preferably about 1-10 micromolar. The predetermined period of time may be about 1 minute to less than about 24 hours, preferably 1-24 hours, and most preferably 3, 5, or 24 hours. The predetermined temperature may be about 4 °C to about 50 °C, preferably about 37 °C.

B. Measuring the Potency of Caspase Cascade Activation

Using a fluorescent plate reader, an initial reading (T=0) is made immediately after addition of the reporter reagent solution, employing excitation and emission at an appropriate wavelength (preferably excitation at 485 nm and emission at 530 nm) to determine the background absorption and/or fluorescence of the control sample. After the incubation, the absorption and/or fluorescence of the sample is measured as above (e.g., at T = 3hr).

Sample Calculation:

The Relative Fluorescence Unit values (RFU) are used to calculate the potency of the test compounds as follows:

$$RFU_{(T=3hr)} - RFU_{(T=0)} = \text{Net RFU}$$

The potency of caspase cascade activation is determined by the ratio of the Net RFU value for a test compound to that of control samples as follows:

$$\frac{\text{Net RFU of test compound}}{\text{Net RFU of control sample}} = \text{Ratio}$$

Preferred test compounds are those indicating a ratio of 2 or greater and most preferably with a measured ratio greater than a statistically significant value calculated as (Ave Control RFU + 4 x $SD_{Control}$) / (Ave Control RFU) for that run. In all embodiments, preferred compounds can also be determined by reference to Tables 1, 2, 3, and 4 which set forth exemplary compounds. Table 2 reports the average data and standard deviation for a number of compounds shown in Table 1. See also Fig. 1 which shows the standard deviation error bar. These results demonstrate that the test compounds that have a ratio around the control value have consistent values and do not lead to false positives. The results also show that compounds with ratios above about 1.5 exhibit wider variation in the numbers they generate. Thus, the assay may give false negatives unless it is run several times.

Table 1

Plate, 88 compounds total							
Position	**Compound Name**	**Compound Indication**	**RFU T=0** (Relative Fluoroscence Units)	**RFU T=3h**	**Net RFU T=3h** [(RFU T=3h) (Control RFU T=0)]	**Ratio** [(Net RFU T=3h) / (Control Net RFU T=3h)]	**Propidium Iodide Ratio**
	Control (DMSO)		500	1103	603	1.0	
	Vinblastine (10 µg/ml)		1293	1001	9511	15.8	Max. ratio = 3.6
E10	Vinblastine sulfate	antineoplastic, tetratogen	928	7880	7380	12.2	1.0
H12	camptothecin	antineoplastic (derivative in clinical use=topotecan)	916	7282	6782	11.2	1.0
C04	colchicine	antineoplastic, gout suppressant	827	6934	6434	10.7	0.9
B09	cytochalasin d	blocks formation contractile microfilaments - multinucleated cells an nuclear extrusion	760	6070	5670	9.2	1.0
G03	puromycin	antineoplastic, antiprotozoal	848	5747	6247	8.7	1.0
D03	gramicidin	antibacterial, ion channel uncoupler	797	5210	4710	7.8	1.0
B11	chloroquins	antimalarial, antiamebic, antifheumatic intercalating agent	736	4624	4124	6.8	0.9
A12	staurosporine	protein kinase C activator	723	4499	3999	6.6	1.0
A02	Actinomycin D	antineoplastic, intercalating	775	4447	3947	6.5	0.9
H11	Podophyllotoxin	antineoplastic analogue is etoposide	732	4300	3800	6.3	0.9
C11	emetine	inhibits RNA, DNA and protein synthesis	742	4267	3767	6.2	1.1
B02	Aklavin hydrochloride	antibiotic e-laorhodomycin group	775	4108	3608	6.0	0.8
H03	Cyclohexamide	inhibits protein synthesis	693	4029	3529	5.9	1.0

Plate, 88 compounds total							
Position	**Compound Name**	**Compound Indication**	**RFU T=0** (Relative Fluoroscence Units)	**RFU T=3h**	**Net RFU T=3h** [(RFU T=3h) (Control RFU T=0)]	**Ratio** [(Net RFU T=3h) / (Control Net RFU T=3h)]	**Propidium Iodide Ratio**
H02	5-azacytidine	antineoplastic, pyrimidine antimetabolte	760	3974	3474	5.8	1.0
F06	rotenone	acaricide, ectoparasticide	607	2741	2241	3.7	0.9
D06	Mechlorethamine	antineoplastic, alkylating agent, cross-links DNA	595	2640	2140	3.5	1.0
B12	doxorubicin	antineoplastic, topo II inhibitor	732	2448	1948	3.2	0.6
H07	Cetylpyridinium chloride	topical anti-infective membrane distuptor	562	2045	1545	2.6	1.0
B03	citrinin	antibiotic Penicillium citinum	555	1996	1496	2.5	0.7
F12	etoposide	antineoplastic, antimitotic	568	2017	1517	2.5	1.0
C10	digitoxin	cardiotonic, cardiotoxic inhibits Na/K ATPase	501	1831	1331	2.2	1.0
G02	etoposide	antineoplastic, antimitotic arrests metaphase inhibits microtubule assembly	565	1807	1307	2.2	0.9
F10	ouabain	cardiotonic, cardiotoxic inhibits Na/K ATPase	543	1737	1237	2.1	0.9
A07	Cadmium acetate	nephrotoxic alpha 1 antitrypsin inhibitor	546	1657	1157	1.9	1.0
B07	Benzethonium chloride	topical anti-infective membrane disruptor	552	1596	1096	1.8	0.9
F09	Mycophenolic acid	antineoplastic	537	1581	1081	1.8	0.9
C09	dicoumarol	anticoagulant, uncoupler of oxidative respiration	488	1541	1041	1.7	1.0

Plate, 88 compounds total							
Position	**Compound Name**	**Compound Indication**	**RFU T=0** (Relative Fluoroscence Units)	**RFU T=3h**	**Net RFU T=3h** [(RFU T=3h) (Control RFU T=0)]	**Ratio** [(Net RFU T=3h) / (Control Net RFU T=3h)]	**Propidium Iodide Ratio**
F05	strychnine	central stimulant, convulsant	531	1453	953	1.6	0.9
G09	cisplatin	antineoplastic, convulsant crosslinks DNA	537	1401	901	1.5	0.9
B04	cytochalasin p	blocks formation contractile microfilaments multinucleated cells and nuclear extrusion	516	1257	757	1.3	0.9
C02	Chlortetracycline	antibacterial, antiamebic, hepatotoxic	491	1257	757	1.3	0.9
D04	griseofulvin	antifungal, inhibits mitosis in metaphase, interacts with microtubules	476	1312	812	1.3	0.9
E02	quinacrine	anthelmintic, antimalarial intercalating agent	577	1288	788	1.3	1.0
E07	thioguanine	antineoplastic, purine antimetabolite	525	1300	800	1.3	0.9
E09	trioxsalen	melanizing agent, photosensitizer, free radical generator	540	1297	797	1.3	0.9
A03	mitomycin C	antineoplastic alkylating	513	1199	699	1.2	1.0
C05	Collatimethate sodium	antibacterial	510	1196	696	1.2	0.9
C07	cytarabine	antineoplastic, antivral antimetabolite, inhibits nucleic acid replication	549	1199	699	1.2	0.9
D09	methotrexate	antineoplastic, antirheumatic, folic acid antagonist	534	1251	751	1.2	0.9
F07	aconitine	gastric anesthetic, antipyrotic, cardiotoxic	507	1221	721	1.2	0.9

Plate, 88 compounds total							
Position	**Compound Name**	**Compound Indication**	**RFU T=0** (Relative Fluoroscence Units)	**RFU T=3h**	**Net RFU T=3h** [(RFU T=3h) (Control RFU T=0)]	**Ratio** [(Net RFU T=3h) / (Control Net RFU T=3h)]	**Propidium Iodide Ratio**
G05	Arcaine sulphate	NO synthase inhibitor, NMDA inhibitor, anti-protozoal	522	1202	702	1.2	1.0
G06	5-fluoro-5'-deoxyuridine	antineoplastic, pyrimidine antimetabolte	525	1215	715	1.2	1.0
G07	Anthraquinone	irritant	510	1221	721	1.2	1.0
H06	aminopterin	antimetabolite, inhibits nucleic acid replication, abortifacient, tetratogen	522	1251	751	1.2	1.0
A04	Methylmethane sulfonate	alkylating agent	494	1190	690	1.1	1.0
A05	Phorbol myristate acetate	tumor promoter PKC activator	510	1135	635	1.1	0.9
A09	2,4-dinitrophenol	uncouples oxidative phosphorylation	494	1184	684	1.1	1.0
B08	busulfan	antineoplastic alkylating agent at guanine	497	1138	638	1.1	1.0
C03	Chloramphenicol	antibacterial, antirickltslal inhibits protein synthesis	482	1138	638	1.1	1.0
C12	Sodium aurothlomalate		525	1184	684	1.1	1.0
D02	5-fluorouracil	antineoplastic, pyrimidine antimetabolte	522	1184	684	1.1	0.9
F04	pentamidine	antiprotozoal, antipneumocytis, inhibits protozoal DNA, RNA phosphollpid and protein synthesis membrane disruptor	488	1160	660	1.1	0.9
G04	picrotoxinin	stimulant, convulsant, GABA receptor antagonist, ichthyotoxin	507	1169	669	1.1	1.0

Plate, 88 compounds total							
Position	Compound Name	Compound Indication	RFU T=0 (Relative Fluoroscence Units)	RFU T=3h	Net RFU T=3h [(RFU T=3h) (Control RFU T=0)]	Ratio [(Net RFU T=3h) / (Control Net RFU T=3h)]	Propidium Iodide Ratio
G11	Zidovudine (AZT)	RT transferase inhibitor, antiviral	494	1148	648	1.1	1.0
H04	azaserine	antineoplstic, amino acid antagonist, inhibits protein and nucleic acid synthesis	488	1138	638	1.1	0.9
H05	p-fluorophenylaline	amino acid antagonist, protein synthesis inhibitor	513	1141	641	1.1	0.9
A06	Benzo (a) pyrene	carcinigen	497	1099	599	1.0	1.0
A11	1,2-dimethylhydrazine hydrochloride	antineoplastic, generate methyl carbanium ions	479	1093	593	1.0	1.0
B10	chlorambucil	antineoplastic tetratogen alklating agent	510	1126	626	1.0	0.9
C06	Cyclophosphamide	antineoplastic alkylating agent	531	1099	599	1.0	0.9
E04	Sisomicin sulfate	antibacterial, binds to ribosomes, nephrotoxic, ototoxic	528	1080	580	1.0	0.9
E05	streptozosin	antineoplastic alkylating agent	528	1093	593	1.0	0.9
E12	amethopterin	methotrexate	488	1120	620	1.0	1.0
G10	antimycin a	antifungal, antiviral, interferes incytochrome oxidation	485	1111	611	1.0	1.0
H09	penicillic acid	toxic metabolite of penicillum sp., antineoplastic, tetratogen, hepatotoxic	637	1108	608	1.0	1.0
F02	acriflavinium (INTRINSIC fluorescence)	anti-infective, intercalating agent, interferes with DNA transcription and replication	24428	22808	22308	0.9	0.9
A10	Sodium fluoroacetate	inhibits critric acid cycle through aconltase	488	1019	519	0.9	1.0

Plate, 88 compounds total							
Position	Compound Name	Compound Indication	RFU T=0 (Relative Fluoroscence Units)	RFU T=3h	Net RFU T=3h [(RFU T=3h) (Control RFU T=0)]	Ratio [(Net RFU T=3h) / (Control Net RFU T=3h)]	Propidium Iodide Ratio
C08	dacarbazine	antineoplastic, alkylating agent, antimetabolite	540	1025	525	0.9	1.0
D05	hydroxyurea	antineoplastic, inhibits ribonucleoside diphosphate reductase	522	1068	568	0.9	1.0
D07	malphalan	antineoplastic, alkylating agent	540	1047	547	0.9	0.9
D08	Mercaptopurine	antineoplastic, purine antimetabolite, inhibits nucleic acid replication	525	1044	544	0.9	1.0
E08	Tobramycin sulfate	antibacterial, tetratogenic, binds to ribosomes, inhibits protein synthesis, nephrotoxic	522	1013	513	0.9	1.0
E11	acetarsol	antiprotozoal, antiphilitic, neurotoxic	504	1050	550	0.9	1.0
G12	taxol	antineoplastic	522	1016	516	0.9	1.0
H08	atractyloside	inhibits oxidative phosphorylation, inhibits adenosine nucleotide transfer	516	1025	525	0.9	1.0
H10	Desmethyldihydrocapsaicin	topical analgesic, depletes Substance P	534	1025	525	0.9	1.0
H08	3-methylcholanthrene	carcinogen DNA binder	546	974	474	0.8	1.1
B05	azathioprine	immunosuppressive, antineoplastic, antiheumatic, antimetabolite	528	986	486	0.8	0.8
B06	bacitracin	antibacterial nephrotoxic nontoxic to humans	534	974	474	0.8	1.1
D12	cytosine B-α-	inhibits DNA synthesis, chain	504	998	498	0.8	0.9

Plate, 88 compounds total							
Position	Compound Name	Compound Indication	RFU T=0 (Relative Fluoroscence Units)	RFU T=3h	Net RFU T=3h [(RFU T=3h) (Control RFU T=0)]	Ratio [(Net RFU T=3h) / (Control Net RFU T=3h)]	Propidium Iodide Ratio
	arabinofuranoside	terminator; anti-cancer					
E03	roxarsone	antibacterial, neurotoxic	516	998	498	0.8	1.0
F03	iproniazid	monoamine oxidase inhibitor, antidepressant hepatotoxic	528	998	498	0.8	1.0
F08	ajmaline	antihypertensive, antiarrhythmic, PAF blocker, cardiotoxic, inhibits glucose uptake	476	998	498	0.8	1.1
G08	carboplatin	antineoplastic, convulsant crosslinks DNA	526	974	474	0.8	1.0
D10	nystatin	antifungal, nephrotoxic alter cell membrane permeability by binding to sterol components	501	940	440	0.7	0.9
D11	Polymyxin b sulfate	antibacterial cationic membrane disruptor	949	952	452	0.7	0.9
F11	cantharidin	irritant, active principle in cantharides and other insect vessicants	486	943	443	0.7	0.9
E06	thimerosal	anti-infective preservative, neurotoxic, membrane disruptor	476	632	132	0.2	1.1

Table 2

		3hr MT220	3 hr MT234	3 hr MT262	3 JD149	3 hr JD150	3 hr JD162	3 hr MT279	3 hr JD168	3 hr JD183	AVE	SDP
	A01										0.0	0.0
Actinomycin D	A02	6.5	12.9	2.9	1.7	3.1	1.7	1.4	1.3	1.7	3.7	3.6
Mitomycin C	A03	1.2	1	0.9	1	1.2	1.3	1.5	1.1	1	1.1	0.2
Methylmethane sulfonate	A04	1.1	1.1	0.8	0.8	1	1.2	1.4	1.2	1	1.1	0.2
phorbol myristate acetate	A05	1.1	1.2	0.8	1.1	1	1.3	1.9	0.9	1.3	1.2	0.3
benzo (a) pyrene	A06	1	0.9	1	1	0.9	1.2	1.5	0.9	0.8	1.0	0.2
Cadmium acetate	A07	1.9	2.3	1	1	1.5	1.3	1.4	1.1	0.9	1.4	0.4
3-methylcholanthrene	A08	0.8	1	0.8	0.9	1.4	1.2	1.4	1.1	0.9	1.1	0.2
2,4-dinitrephenol	A09	1.1	0.9	1	0.8	0.8	1	1.5	1	0.8	1.0	0.2
sodium fluoroacetate	A10	0.9	1	1.1	1	1	1.3	1.3	1.1	1	1.1	0.1
1,2-dimethylhydrazine hydrochloride	A11	1	0.8	1.1	1	0.8	1.3	1.4	0.7	1.1	1.0	0.2
Staurosporine	A12	6.6	12.2	4.2	4.3	4	3.7	3.2	2.5	5.3	5.1	2.7
	B01											
aklavin hydrochloride	B02	6	10.9	2.6	1.8	2.5	1.9	1.8	1.5	2.4	3.5	2.9
Citrinin	B03	2.5	1.1	1.3	1.1	2	1.3	1.4	1.1	1.2	1.4	0.5
Cyclochalasin p	B04	1.3	1.1	0.8	0.9	0.9	1.1	1.4	1.2	1.1	1.1	0.2

		3hr MT220	3 hr MT234	3 hr MT262	3 JD149	3 hr JD150	3 hr JD162	3 hr MT279	3 hr JD168	3 hr JD183	AVE	SDP
Azathioprine	B05	0.8	1.1	1	0.9	0.9	1.1	1.2	1	1.1	1.0	0.1
Bacitracin	B06	0.8	1	1	1	0.9	1.1	1.6	1	0.9	1.0	0.2
Benzethorium chloride	B07	1.8	1.7	0.9	1.1	1.3	1.5	1.4	1	1.1	1.3	0.3
Busulfan	B08	1.1	1.1	0.8	0.9	0.6	1.3	1.2	1.1	1	1.0	0.2
Cytochalasin d	B09	9.2	1.7	1.9	1.2	3.3	2.5	1.7	1.4	3.4	2.9	2.3
Chlorambucil	B10	1	0.9	1.1	0.9	1	1	1.3	0.9	1	1.0	0.1
Chloroquine	B11	6.8	6.2	1.1	0.8	0.5	1.3	1.2	0.8	1.1	2.2	2.3
Doxorubicin	B12	3.2	6.1	1.1	1.7	1.9	1.8	1.9	1.3	1.4	2.3	1.5
	C01											
Chlortetracycline	C02	1.3	1.2	0.7	1	0.9	1.1	1.4	1.1	1.1	1.1	0.2
Chloroamphenical	C03	1.1	1	0.8	1	0.9	1	1.4	1.1	1.1	1.0	0.2
Colchicine	C04	10.7	6.3	3.7	2.7	3.7	4.7	3.4	2.9	2.1	4.5	2.5
Colistimethate sodium	C05	1.2	1	0.9	0.7	0.9	1.2	1.3	1	1.2	1.0	0.2
Cyclophosphanate	C06	1	1	0.9	1	1.1	1.2	1.2	1.1	1	1.1	0.1
Cytarabine	C07	1.2	1.3	1.9	2.3	1.2	4.6	4.3	3.4	1.1	2.4	1.3
Dacarbazine	C08	0.9	1	0.8	1	0.9	1.2	1.3	0.9	0.9	1.0	0.2
Dicoumarol	C09	1.7	2	1.5	0.7	0.6	1.2	1.5	1	0.9	1.2	0.4
Digitoxin	C10	2.2	1.9	1.7	0.9	1.6	1.4	1.3	0.8	1.1	1.4	0.4

		3hr MT220	3 hr MT234	3 hr MT262	3 JD149	3 hr JD150	3 hr JD162	3 hr MT279	3 hr JD168	3 hr JD183	AVE	SDP
Emetine	C11	6.2	8.6	3.6	1.8	2.6	2.6	2.7	1.2	5.2	3.8	2.3
sodium aurothlomalate	C12	1.1	1.1	0.8	1.3	0.8	1	1.4	0.9	1	1.0	0.2
	D01											
5-fluorouracil	D02	1.1	1.1	0.8	1	1	1.1	1.2	1	1.2	1.1	0.1
Gramicidin	D03	7.8	3.9	6.1	7.1	3.6	13.3	7.9	7	2.1	6.5	3.1
Griscofulvin	D04	1.3	1.2	0.7	0.8	1.1	1	1.6	1	1.3	1.1	0.3
Hydroxyurea	D05	0.9	1.3	0.8	0.8	0.8	1.3	1.4	1	1.2	1.1	0.2
Mechlorethamine	D06	3.5	8	1.1	2.1	2.4	11.8	3	0.8	1.1	3.8	3.5
Malphalan	D07	0.9	1	0.9	1	1.1	1.1	1.2	1	1	1.0	0.1
Mercaplopurine	D08	0.9	1	0.8	0.8	1	1.2	1.1	0.9	1	1.0	0.1
Methotrexate	D09	1.2	1	1	1.3	1.1	1.4	1.3	1.1	0.9	1.1	0.2
Nystatin	D10	0.7	0.8	0.9	0.8	0.9	1.1	1.2	0.9	1.1	0.9	0.2
Polymyxin b sulfate	D11	0.7	0.7	1.1	0.7	0.9	1.2	1.2	0.8	1.1	0.9	0.2
Cytosine B-a arabinofuranoside	D12	0.8	1	1.7	2.8	1.4	4	5.2	3.7	0.9	2.4	1.5
10 μM VINBLASTINE OR 10 μm cisplatin	E01	15.8	10.2	10.8	6.8	6.5	9.9	8.1	4.8	-	9.1	3.2
Quinacrine	E02	1.3	1.3	0.7	1	0.9	1.1	1.6	1.1	1	1.1	0.2
Roxarsone	E03	0.8	1	0.7	0.9	0.8	1.1	1	1.1	0.9	0.9	0.1

		3hr MT220	3 hr MT234	3 hr MT262	3 JD149	3 hr JD150	3 hr JD162	3 hr MT279	3 hr JD168	3 hr JD183	AVE	SDP
Sisomicin sulfate	E04	1	1.1	0.8	0.9	0.9	1.3	1.4	1	1.1	1.1	0.2
Streptozosin	E05	1	1.1	0.8	0.7	0.8	1.2	1.3	0.9	1.1	1.0	0.2
Thimerosal	E06	0.2	0.2	0.4	0.5	0.6	0.3	0.8	0.4	0.4	0.4	0.2
Thioguanine	E07	1.3	1.1	0.8	0.8	0.9	1.1	1.2	0.9	0.9	1.0	0.2
Tobramycin sulfate	E08	0.9	1	0.8	0.8	0.6	1.2	1.1	0.9	1	0.9	0.2
Trioxsalen	E09	1.3	0.9	0.9	0.8	1	1.2	1.3	0.8	1	1.0	0.2
Vinblastine sulfate	E10	12.2	6.1	7.1	6	6.4	9.6	6.9	6.1	3.4	7.1	2.3
Acetarsol	E11	0.9	0.8	1	0.7	0.7	1.3	1.3	0.7	1	0.9	0.2
Amethopterin	E12	1	0.8	0.7	1.3	1.2	1.4	1.5	1.3	0.9	1.1	0.3
	F01											
Acriflavinium (INTRINSINC fluorescence)	F02	37	28.8	24.6	7.6	6.6	21.6	31.5	16.3	21.8	21.8	9.7
Iproriazid	F03	0.8	1	0.9	0.9	0.8	1.3	1.2	0.9	1.1	1.0	0.2
Pentamidine	F04	1.1	1	0.8	0.8	0.9	1.3	1.3	1	1.1	1.0	0.2
Strychnine	F05	1.6	1.2	0.9	0.8	0.9	1.4	1.2	1.1	1.2	1.1	0.2
Rotenone	F06	3.7	3.1	2.7	1.8	3	3.1	2.8	1.9	1.8	2.7	0.6
Aconitine	F07	1.2	1.1	1	0.8	0.9	1.3	1.3	0.9	1.1	1.1	0.2
Ajmaline	F08	0.8	1	0.8	0.9	0.8	1.1	1.2	0.8	1.1	0.9	0.1

		3hr MT220	3 hr MT234	3 hr MT262	3 JD149	3 hr JD150	3 hr JD162	3 hr MT279	3 hr JD168	3 hr JD183	AVE	SDP
Mycophenolic acid	F09	1.8	1	0.8	0.7	0.9	1.1	1.2	0.8	0.9	1.0	0.3
Ouabain	F10	2.1	1.4	1.3	0.8	1.5	1.4	1.2	0.9	1	1.3	0.4
Canthandin	F11	0.7	0.8	1.1	1.2	3.1	16.1	1.3	0.8	0.9	2.9	4.7
Eloposide	F12	2.5	4.1	7.3	8.1	7.4	13.8	8.7	9.8	0.9	7.0	3.7
	G01											
Etoposide	G02	2.2	1.9	6.2	6.6	5.8	11.7	10.4	8.5	1	6.0	3.6
Puromycin	G03	8.7	10.4	3.8	1.6	3.3	2.7	1.9	1.7	7.6	4.6	3.2
Picroloxinin	G04	1.1	1.1	0.9	1	0.9	1	1.3	1.2	1	1.1	0.1
Arcaine sulphate	G05	1.2	1	0.9	0.8	0.8	1.2	1.2	1	1.1	1.0	0.2
5-fluoro-5'-deoxyuridine	G06	1.2	1	1	0.8	0.8	1	1.4	0.9	0.9	1.0	0.2
Anthraquinone	G07	1.2	1.1	0.8	0.8	0.6	1.2	1.3	0.9	1.2	1.0	0.2
Carboplatin	G08	0.8	0.9	0.8	0.5	0.8	1.2	1.2	1	0.9	0.9	0.2
Cisplatin	G09	1.5	1	0.7	0.7	0.8	1.2	1.2	0.9	0.9	1.0	0.3
Antimycin a	G10	1	0.9	0.9	0.8	0.8	1.2	1.4	0.8	0.9	1.0	0.2
Zidovudine (AZT)	G11	1.1	0.8	0.9	0.8	0.6	1.2	1.1	0.9	1.1	0.9	0.2
Taxol	G12	0.9	0.8	0.6	1	0.8	1.2	1.3	0.8	0.8	0.9	0.2
	H01											
5-azacylidine	H02	5.8	10.1	4	6.5	5.2	12.3	2.2	2.9	1.2	5.6	3.4

		3hr MT220	3 hr MT234	3 hr MT262	3 JD149	3 hr JD150	3 hr JD162	3 hr MT279	3 hr JD168	3 hr JD183	AVE	SDP
Cyclohexamide	H03	5.9	4.9	6.5	4.5	2.1	7.4	6.8	4.7	5.5	5.4	1.5
Azasenne	H04	1.1	1.2	1	0.8	0.7	1.1	1.3	0.8	1	1.0	0.2
p-fluorophenylaline	H05	1.1	1	1	0.7	0.9	1	1.2	0.8	1.3	1.0	0.2
Aminopterin	H06	1.2	1.2	1.2	1.5	1.6	1.5	1.5	1	0.8	1.3	0.3
cefylpyridirium chloride	H07	2.6	3.5	1.4	0.8	0.8	3.5	2.3	1.1	2	2.0	1.0
Atrctyloside	H08	0.9	1	0.8	0.8	0.5	1	1.2	0.9	0.9	0.9	0.2
penicillic acid	H09	1	1	0.8	0.9	0.6	0.9	1.2	0.8	0.8	0.9	0.2
Desmethyldihydro-capsaicin	H10	0.9	0.9	1	0.8	0.6	1.1	1.5	0.7	1.1	1.0	0.2
Podophyllotoxin	H11	6.3	5	4.3	1.9	4.1	3.2	2.3	1.5	1.5	3.3	1.6
Camptothecin	H12	11.2	10.9	10.7	8.8	7.1	15.4	17.9	10.9	4.1	10.8	3.9

Table 3

Compounds with ratio of about 2 or greater are shown						
	Compound Name	**Compound Indication**	**RFU T=0** (Relative Fluorescence Units)	**RFU T=3h**	**Net RFU T=3h** [(RFU T=3h) / (Control RFU T=0)]	**Ratio** [(Not RFU T=3h) / (Control Net RFU T-3h)]
Generic Library Plate 1, position	**Control (DMSO)**		456	1357	901	1.0
	vinblastine (10 µg/ml)		1109	6520	6064	6.7
F06	benzethonium chloride	Topical anti-infective	549	2863	2407	2.7
F02	baclofen	Muscle relaxant (skeletal)	519	1376	920	2.7
G08	bithionolate sodium	anthelmintic; antiseptic	577	2805	2349	2.6
E03	apomorphine	emetic	595	2704	2248	2.5
D07	amphotericin B	antifungal	949	2411	1955	2.2
D02	aminopterin	rodenticide	549	1856	1400	1.6
Generic Library Plate 2, position	**Control (DMSO)**		408	1212	803	1.0
	vinblastine (10 µg/ml)		605	7786	7378	9.2
E07	cytarabine	antineoplastic; antiviral	528	3687	3279	4.1
D08	colchicine	gout suppressant	452	3427	3019	3.8
F02	dehydrocholic acid	choleretic	397	1318	910	3.3
G07	dienestrol	estrogen	439	2381	1973	2.5
A08	cetylpyridinium chloride	topical anti-infective	406	1862	1454	1.8

Compounds with ratio of about 2 or greater are shown						
	Compound Name	**Compound Indication**	**RFU T=0** (Relative Fluorescence Units)	**RFU T=3h**	**Net RFU T=3h** [(RFU T=3h) / (Control RFU T=0)]	**Ratio** [(Not RFU T=3h) / (Control Net RFU T-3h)]
Generics Library Plate 3, position	**Control (DMSO)**		387	841	454	1.0
	vinblastine (10 µg/ml)		463	7152	6766	14.9
F05	gentian violet	antibacterial: anthelmintic (nematodes)	1202	10389	10002	22.0
F07	gramicidin	antibacterial	821	7480	7093	15.6
C10	estradiol valerate	estrogen	589	5286	4899	10.8
G10	hexylresorcinol	anthelmintic (nematodes); topical antiseptic	449	3021	2634	5.8
A11	disulfiram	alcohol deterrent	403	1553	1166	2.6
F02	gamma-aminobutyric acid	antihypertensive	372	882	495	2.4
B08	emetine hydrochloride	antiamebic; inhibits RNA, DNA and protein synthesis	394	1291	904	2.0
E10	furosemide	diuretic; antihypertensive	403	1297	910	2.0
B04	doxycycline	antibacterial	391	1230	843	1.9
Generics Plate 4, position	**Control (DMSO)**		587	1963	1376	1.0
	vinblastine (10 µg/ml)		2320	14769	14181	10.3
F02	methotrexate	antineoplastic;	607	1972	1385	3.2

Compounds with ratio of about 2 or greater are shown						
	Compound Name	**Compound Indication**	**RFU T=0** (Relative Fluorescence Units)	**RFU T=3h**	**Net RFU T=3h** [(RFU T=3h) / (Control RFU T=0)]	**Ratio** [(Not RFU T=3h) / (Control Net RFU T-3h)]
		antirheumatic folic acid antagonist				
A02	hydroxyprogester one caproate	progestogen	794	4151	3564	2.6
D03	mechlorethamine	antineoplastic; tetratogen alkylating agent: crosslinks DNA	751	3888	3301	2.4
Generics Plate 5, position	**Control (DMSO)**		576	1710	1134	1.0
	Vinblastine (10 µg/ml)		2603	14194	13633	10.9
A11	ROTENONE	Acaricide, ectoparasiticide inhibits NADH-2 oxidation to NAD	674	4261	3700	3.0
F02	TOLBUTAMIDE	Antidiabetic agent	601	1792	1231	3.0
C02	STROPHANTHIDIN	Glycoside (cardiac)	736	3894	3333	2.7
G11	TYROTHRICIN	Topical antibacterial	671	3736	3175	2.5
Generics Library Plate 7, position	**Control (DMSO)**		**588**	2642	1875	1.0
	Vinblastine (10 µg/ml)		1320	11579	10991	5.9
F09	PUROMYCIN	Antineoplastic;	1950	15977	15389	8.2

Compounds with ratio of about 2 or greater are shown						
	Compound Name	**Compound Indication**	**RFU T=0** (Relative Fluorescence Units)	**RFU T=3h**	**Net RFU T=3h** [(RFU T=3h) / (Control RFU T=0)]	**Ratio** [(Not RFU T=3h) / (Control Net RFU T-3h)]
		antiprotozoal protein synthesis inhibitor				
F02	PIMETHIXENE	Histamine, H1-receptor antagonist	580	2597	2009	4.5
Generics Library Plate 8, position	**Control (DMSO)**		**533**	2561	2028	1.0
	Vinblastine (10 µg/ml)		1258	11795	11262	5.6
F02	ESTRADIOL DISULFATE, POTASSIUM SALT	estrogen	565	2417	1884	4.3
A10	ATROVENETIN	antibiotic	696	5643	5110	2.5
B02	ETOPOSIDE	antineoplastic, antimitotic	772	5512	4979	2.5
Generics Library Plate 9, position	**Control (DMSO)**		**517**	1531	1014	1.0
	Vinblastine (10 µg/ml)		1513	12860	12343	12.2
H09	GOSSYPOL - ACETIC ACID	inhibits pepsinigen conversion to pepsin	632	3922	3405	3.4
F02	7-CHLOROKYNURENIC ACID	NMDA receptor antagonist (gly site)	555	1593	1076	2.9

Compounds with ratio of about 2 or greater are shown						
	Compound Name	**Compound Indication**	**RFU T=0** (Relative Fluorescence Units)	**RFU T=3h**	**Net RFU T=3h** [(RFU T=3h) / (Control RFU T=0)]	**Ratio** [(Not RFU T=3h) / (Control Net RFU T-3h)]
Generics Library Plate 10, position	**Control (DMSO)**		485	1319	834	1.0
	Vinblastine (10 µg/ml)		1523	10275	9790	11.7
A08	5-AZACYTIDINE	Antineoplastic	1178	8817	8332	10.0
A02	AKLAVIN HYDROCHLORIDE	antibiotic J Antibiotics 30:88(1977)	1126	7715	7230	8.7
F02	BENZAMIL HYDROCHLORIDE	Sodium channel inhibitor	739	4685	4200	6.3
H09	PODOPHYLLOTOXIN	Antineoplastic	769	5283	4798	5.8
A09	CYCLOHEXIMIDE	inhibits protein synthesis	653	4383	3898	4.7
E08	AMIODARONE	Adrenergic receptor agonist; coronary vasodilator calcium channel (L type) blocker	549	3662	3177	3.8
D10	1-(5-ISOQUINOLINESULFONYL)-2-METHYLPIPERAZINE HYDROCHLORIDE	PKC inhibitor [Biochem Biophys Res Comm 125: 258 (1984)]	598	3089	2604	3.1
H02	VERAPAMIL HYDROCHLORIDE	Adrenegic receptor blocker, calcium channel blocker, coronary vasodilator, antiarrhythmic	571	2695	2210	2.6
G02	LOPERAMIDE	Calcium channel blocker	540	2023	1538	1.8

Compounds with ratio of about 2 or greater are shown						
	Compound Name	**Compound Indication**	**RFU T=0** (Relative Fluorescence Units)	**RFU T=3h**	**Net RFU T=3h** [(RFU T=3h) / (Control RFU T=0)]	**Ratio** [(Not RFU T=3h) / (Control Net RFU T-3h)]
EXAMPLE OF INACTIVE COMPOUNDS WITH A RATIO OF ABOUT 1, FROM PLATE 10						
H04	N-AMINOEXYL-5-CHLORONAPHTH-1-YLSULFONAMIDE HYDROCHLORIDE [W-7]	Calmodulin antagonist [PNAS 78:4354 (1981)]	516	1398	913	1.1
H07	6-METHOXY-1,2,3,4-TETRAHYDRO-9H-PYRIDO[3,4b]INDOLE	MAO inhibitor [J Pharm Sci 57:269 (1968)]	476	1410	925	1.1
H08	METOLAZONE	Diuretic antihypertensive	473	1395	910	1.1
H10	NIPECOTIC ACID	GABA uptake inhibitor [J Neurochem 25:797 (1975)]	482	1395	910	1.1
A10	AZASERINE	Therap cat; antineoplastic aminoacid antagonist: inhibits protein and nucleic acid synthesis	479	1328	843	1.0
B06	CARBIDOPA	Decarboxylase inhibitor; adjunct to levodopa in parkinsonism	488	1303	818	1.0
B10	t-BOCNLEU-LEU-PHE	Chemotaxis inhibitor	510	1352	867	1.0
C06	KYOTORPHIN ACETATE	Analgesic	482	1288	803	1.0
C07	[D-ARG]KYOTORPHIN ACETATE	Analgesic	488	1328	843	1.0

Table 4

Natural Products Library Compounds, 640 compounds total, 80 compounds per plate,						
Compounds with ratio of about 2 or greater are shown						
	Compound Name	MS ID#	RFU T=0 (Relative Fluorescence Units)	RFU T=3h	Net RFU T=3h [(RFU T=3h) / (Control RFU T=0)]	Ratio [(Not RFU T=3h) / (Control Net RFU T-3h)]
Natural Prod. Plate 1, position	Control (DMSO)		434	775	342	1.0
	vinblastine (10 g/ml)		706	5245	4811	14.1
G02	7-DESACETOXY-6-7-DEHYROGEDUNIN	100389	592	2927	2493	7.3
C06	6,2',3'-TRIHYDROXY-7,4'-DIMETHOXYISOFLAVAN	201410	504	1242	808	2.4
E06	3-DEOXY-7-OXODIHYDROGEDUNIN	100406	473	1193	759	2.2
F06	2',3'-DIMETHOXY-4'-BENZYLOXYFLAVAN	201401	473	1041	607	1.8
Natural Prod. Plate 2, position	Control (DMSO)		449	720	271	1.0
	Vinblastine (10 g/ml)		756	4711	4263	15.7
A06	ANTHOTHECOL	100005	720	4081	3633	13.4
B04	CHUKRASIN	100010	641	2933	2485	9.2
B03	CEDRELONE	100009	470	1322	874	3.2
C10	HETEROPEUCENIN, METHYLETHER	100532	443	1013	566	2.1
Natural						

Natural Products Library Compounds, 640 compounds total, 80 compounds per plate,						
Compounds with ratio of about 2 or greater are shown						
	Compound Name	MS ID#	RFU T=0 (Relative Fluorescence Units)	RFU T=3h	Net RFU T=3h [(RFU T=3h) / (Control RFU T=0)]	Ratio [(Not RFU T=3h) / (Control Net RFU T-3h)]
Prod. Plate 3, position	Control (DMSO)		507	1992	1486	1.0
	Vinblastine (10 g/ml)		1147	13630	13124	8.8
B03	GAMBOGIC ACID	200026	1532	16490	15984	10.8
F04	DIACETYLDIDEISOVAL ERYL-RHODOMYRTOXIN	211118	1126	10584	10078	6.8
H10	PODOPHYLLOTOXIN	2300332	916	9983	9477	6.4
G03	SERICETIN DIACETATE	201584	818	7758	7252	4.9
B05	GAMBOGINIC ACID METHYLESTER	210674	742	7523	7017	4.7
B04	GAMBOGIC ACID AMIDE	201522	742	6861	6355	4.3
F02	PSOROMIC ACID	200040	519	2188	1682	4.2
E11	POMIFERIN	201580	629	6205	5699	3.8
G10	SUMATROL ACETATE	200822	595	5475	4969	3.3
A03	BRAZILIN	200012	665	4709	4203	2.8
C06	ISOGINKGETIN	200433	647	4425	3919	2.6
D05	LONCHOCARPIC ACID DIACETATE	203015	552	3937	3431	2.3
F07	ROTENONE	200013	571	3949	3443	2.3
E02	OSAJIN	201595	528	3568	3062	2.1
Natural Prod. Plate 4, position	Control (DMSO)		465	1708	1243	1.0

Natural Products Library Compounds, 640 compounds total, 80 compounds per plate,						
Compounds with ratio of about 2 or greater are shown						
	Compound Name	**MS ID#**	**RFU T=0** (Relative Fluorescence Units)	**RFU T=3h**	**Net RFU T=3h** [(RFU T=3h) / (Control RFU T=0)]	**Ratio** [(Not RFU T=3h) / (Control Net RFU T-3h)]
	Viblastine (10 g/ml)		1331	11202	10737	8.6
A11	CELASTROL	300145	1465	13765	13300	10.7
F02	ORSELLINIC ACID ETHYLESTER	200002	418	1630	1165	3.9
D03	GITOXIGENIN DIACETATE	100584	510	3406	2941	2.4
H03	STROPHANTHIDIN	100291	482	3443	2978	2.4
B05	CYMARIN	100698	504	3284	2619	2.3
H04	STROPHANTHIDIN ACETATE	100292	510	3339	2874	2.3
F05	PERIPLOCYMARIN	100588	510	3144	2679	2.2
B10	DIGOXIN	100707	464	3064	2599	2.1
C02	EMICYMARIN	100605	4880	3031	2566	2.1
H06	STROPHANTHIDINIC ACID LACTONE ACETATE	100749	501	3043	2578	2.1
G03	SARMENTOGENIN	100682	421	2878	2413	1.9
Natural Prod. Plate 5, position	**Control (DMSO)**		443	1211	768	1.0
	Vinblastine (10 g/ml)		1088	9134	8691	11.3
D07	EMETINE	1500272	1172	8366	7924	10.3
A07	AKLAVIN HYDROCHLORIDE	200024	1160	8060	7618	9.9
F02	MELATONIN	1500690	519	1352	910	2.6
C10	CHELIDONINE(+)	1500824	543	2442	2000	2.6

Natural Products Library Compounds, 640 compounds total, 80 compounds per plate,						
Compounds with ratio of about 2 or greater are shown						
	Compound Name	MS ID#	RFU T=0 (Relative Fluorescence Units)	RFU T=3h	Net RFU T=3h [(RFU T=3h) / (Control RFU T=0)]	Ratio [(Not RFU T=3h) / (Control Net RFU T-3h)]
F06	NORELEAGNINE	1500939	552	2381	1939	2.5
C06	CALYCANTHINE	500061	559	2091	1649	2.1
D06	DICTAMINE	100541	494	1984	1542	2.0
Natural Prod. Plate 6, position	Control (DMSO)		495	1159	664	1.0
	Vinblastine (10 g/ml)		1539	8682	8187	12.3
B09	DIHYDROGAMBOGIC ACID	240474	2121	10709	10214	15.4
C05	GAMBOGIC ACID DIETHYLAMIDE	211216	2014	9849	9354	14.1
B05	CRASSIN ACETATE	300037	800	3699	3204	4.8
H05	TETRAHYDROGAMBO GIC ACID	240479	934	3357	2862	4.3
B07	DECAHYDROGAMBOGIC ACID	240473	699	2844	2349	3.5
E04	DEOXODEOXYDIHYDROGEDUNIN	100218	589	2670	2175	3.3
F02	1(2)a-EPOXY-DEACETOXYDIHYDROGEDUNIN	100233	555	1474	979	2.7
G03	MEXICANOLIDE ENOL ACETATE	100145	629	1828	1333	2.0
Natural Prod. Plate 7, position	Control (DMSO)		543	2440	1897	1.0
	Vinblastine (10 g/ml)		1247	13617	13074	6.9
C02	4,4'-DIMETHOXYDALBERGIONE	201448	1975	17250	16707	8.8

Natural Products Library Compounds, 640 compounds total, 80 compounds per plate,						
Compounds with ratio of about 2 or greater are shown						
	Compound Name	MS ID#	RFU T=0 (Relative Fluorescence Units)	RFU T=3h	Net RFU T=3h [(RFU T=3h) / (Control RFU T=0)]	Ratio [(Not RFU T=3h) / (Control Net RFU T-3h)]
D06	METHYL CAMBOGINATE	240475	1584	14821	14278	7.5
A04	OBTUSAQUINONE	200091	836	10310	9767	5.1
G07	COLCHICINE	1500205	781	9223	8680	4.6
F02	BRAZILIN, TRIMETHYL ETHER	240943	537	2451	1908	4.6
F11	ISORHODOMYRTOXIN	300219	607	5420	4877	2.6
F09	JUGLONE	300038	693	5372	4829	2.5
B05	RESISTOMYCINOLIDE	201117	1953	4758	4215	2.2
Natural Prod. Plate 8, position	Control (DMSO)		488	1847	1358	1.0
	Vinblastine (10 g/ml)		1491	11823	11335	8.3
E08	CUCUMIN	1800014	986	7325	6837	5.0
B08	PODOPHYLLOTOXIN GLYCOSIDE	1500903	839	6000	5512	4.1
F02	DIHYDROCINCHONIDINE	1800056	522	2011	1523	3.9
G11	LEODIN DIACETATE	300516	714	5549	5061	3.7
E09	18a-GLYCYRRHETIC ACID	1800021	671	4044	3556	2.6

Table 1 indicates the results of the propidium iodide test described in Example 1 herein, wherein cells that show a propidium iodide ratio of about 1 are viable cells having an intact cell membrane. Table 1 provides evidence for the effectiveness of the present invention for the purpose of identifying potentially therapeutically effective antineoplastic agents. By contrast to methods previously known in the art, wherein non-viable cells were used to measure apoptosis, the method of the present invention detects therapeutically important caspase activator drugs, ignores non-specific cell poisons, differentiates among known antineoplastic agents such as p53-dependent agents vs. non-p53 independent agents and rapid caspase activator agents vs. other antineoplastic agents. Thus, the method offers important advantages regarding therapeutic relevance and selectivity in the discovery of new antineoplastic agents, over currently available methods.

Table 3 summarizes the results of a library of 800 generic compounds, using 80 compounds per plate. Potentially therapeutically effective antineoplastic agents identified in this library, having a ratio greater than about 2 are shown. Representative samples of inactive compounds, which do not have a ratio greater than about 2, are also shown.

Table 4 shows the results from a library of 640 natural products, tested using 80 compounds per plate. Potentially therapeutically effective antineoplastic agents identified in this library, having a ratio greater than about 2 are shown.

Thus, the present assay identifies the following compounds as activators of the caspase cascade having a potency ratio of greater than about 2 in viable cultured eukaryotic cells having an intact cell membrane: aklavin hydrochloride, citrinin, cytochalasin d, chloroquine, gramicidin, rotenone, cetylpyridinium chloride, staurosporine, emetine, etoposide, digitoxin, ouabain, benzethonium chloride, baclofen, bithinolate sodium, apomorphine, amphotericin B, colchicine, dehydrocholic acid, dienestrol, gentian violet, hexylresorcinol, disulfiram, gamma aminobutyric acid, furosemide, hydroxyprogesterone caproate, mechlorethamine, oxyphenbutazone, strophanthidin, tyrothricin, pimethixene, estradiol disulfate

potassium salt, atrovenetin, gossypol-acetic acid, 7-chlorokynurenic acid, benzamil hydrochloride, amiodarone, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine hydrochloride, verapamil hydrochloride, 7-desacetoxy-6,7-dehydrogedunin, 6,2',3'-trihydroxy-7,4'-dimethoxyisoflavan, anthothecol, chukrasin, cedrelone, heteropeucenin methyl ether, gambogic acid, diacetyldideisovaleryl-rhodomyrtoxin, sericetin diacetate, gamboginic acid methyl ester, gambogic acid amide, pomiferin, sumatrol acetate, brazilin, lonchocarpic acid diacetate, osajin, celastrol, orsellinic acid ethyl ester, gitoxigenin diacetate, strophanthidin, cymarin, stophanthidin acetate, periplocymarin, digoxin, emicymarin, strophanthidinic acid lactone acetate, melatonin, chelidonine, norleagnine, calycanthine dictamine, dihydrogambogic acid, gambogic acid diethyl amide, crassin acetate, tetrahydrogambogic acid, decahydrogambogic acid, deoxodeoxydihydrogedunin, 1-(2-α-epoxy)-deacetoxydihydrogedunin, mexicanolide enol acetate, 4,4'-dimethoxydalberginone, methyl gamboginate, obtusaquinone, brazilin trimethyl ether, isorhodomyrtoxin, juglone, resistomycinolide, cucumin, dihydrocinchonidin, leodin diacetate, and 18α-glycyrrhetic acid.

The identification of some of the above and other compounds that possess the requisite activities are set forth in the Examples. Compounds whose identification is not explicitly exemplified can be identified by routine modification of one or more methods described in detail in the Examples by appropriately modifying conditions without undue experimentation.

C. Formulation and Administration of the Compositions

Effective concentrations of one or more of the caspase cascade activators or pharmaceutically acceptable salts, or prodrugs thereof are mixed with a suitable pharmaceutical carrier or vehicle. In instances in which the compounds exhibit insufficient solubility, methods for solubilizing compounds may be used. Such methods are known to those of skill in this art, and include, but are not

limited to, using solubilizers, such as dimethylsulfoxide (DMSO), surfactants, such as polysorbate 80, dissolution in aqueous sodium bicarbonate, or use of transdermal penetration enhancers (e.g., AZONE ™).

Upon mixing or adding the antineoplastic compound(s) with a suitable carrier or vehicle, the resulting mixture may be a solution, suspension, emulsion or the like. The form of the resulting mixture depends upon a number of factors, including the intended mode of administration and the solubility of the compounds in the selected carrier or vehicle.

Pharmaceutical carriers or vehicles suitable for administration of the compounds provided herein include any such carriers known to those skilled in the art to be suitable for the particular mode of administration. In addition, the compounds may be formulated as the sole pharmaceutically active ingredient in the composition or may be combined with other active ingredients.

The active compounds can be administered by any appropriate route, for example, bucally, intransally, orally, parenterally, intravenously, intradermally, subcutaneously, or topically, in liquid, semi-liquid, or solid form formulated in a manner suitable for each route of administration. Preferred modes of administration include oral, nasal and parenteral modes of administration.

The active compound is included in the pharmaceutically acceptable carrier in an amount sufficient to exert a therapeutically useful effect in the absence of undesirable side effects on the patient treated. The concentration of active compound in the drug composition will depend on absorption, inactivation and excretion rates of the active compound, the dosage schedule, and amount administered as well as other factors known to those of skill in the art.

Typically, a therapeutically effective dosage should produce a serum concentration of active ingredient from about about 50-100 pg/ml to 0.1 mg/ml. The pharmaceutical composition typically should provide a dosage of from about 0.01 mg to about 10 mg of compound per kilogram of body weight per day. The active ingredient may be administered at once, or may be divided into a number of smaller doses to be administered at selected intervals of time. It is understood

that the precise dosage and duration of treatment is a function of the disease being treated and may be determined empirically using known testing protocols or by extrapolation from *in vivo* or *in vitro* test data. It is to be noted that concentrations and dosage values may also vary with the severity of the condition to be alleviated. It is to be further understood that for any particular subject, specific dosage regimens should be adjusted over time according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions, and that the concentration ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed compositions.

If oral administration is desired, the compound may be provided in a composition that protects it from the acidic environment of the stomach. For example, the composition can be formulated in an enteric coating (*e.g.* with hydroxypropylmethyl cellulose phthalate) that maintains its integrity in the stomach and releases the active compound in the intestine (See *Remington's Pharmaceutical Sciences*, Osol, A., ed., Mack Publishing Co. (1980)). The composition may also be formulated in combination with an antacid or other such ingredient.

Oral compositions will generally include an inert diluent or an edible carrier and may be compressed into tablets or enclosed in gelatin capsules. For the purpose of oral therapeutic administration, the active compound or compounds can be incorporated with excipients and used in the form of tablets, capsules or troches. Pharmaceutically compatible binding agents and adjuvant materials can be included as part of the composition.

The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder, such as microcrystalline cellulose, gum tragacanth and gelatin; an excipient such as starch and lactose; a disintegrating agent such as, but not limited to, alginic acid and corn starch; a lubricant such as, but not limited to, magnesium stearate; a glidant, such as, but not limited to, colloidal silicon dioxide; a sweetening agent such as

sucrose or saccharin; and a flavoring agent such as peppermint, methyl salicylate, or fruit flavoring.

When the dosage unit form is a capsule, it can contain, in addition to material of the above type, a liquid carrier such as a fatty oil. In addition, dosage unit forms can contain various other materials that modify the physical form of the dosage unit, for example, coatings of sugar and other enteric agents. The compounds can also be administered as a component of an elixir, suspension, syrup, wafer, chewing gum or the like. A syrup may contain, in addition to the active compounds, sucrose as a sweetening agent and certain preservatives, dyes and colorings and flavors.

The active material can also be mixed with other active materials that do not impair the desired action, or with materials that supplement the desired action, such as antacids, H2 blockers, and antimetabolites. For example, if the compound is used for treating colon cancer, it may be used with other antineoplastic agents.

Solutions or suspensions used for parenteral, intradermal, subcutaneous, intranasal or topical application can include any of the following components: a sterile diluent, such as water for injection, saline solution, fixed oil, polyethylene glycol, glycerin, propylene glycol or other synthetic solvent; antimicrobial agents, such as benzyl alcohol and methyl parabens; antioxidants, such as ascorbic acid and sodium bisulfite; chelating agents such as ethylenediaminotetraacetic acid (EDTA); buffers, such as acetate, citrates, and phosphates; and agents for the adjustment of tonicity such as sodium chloride or dextrose. Parenteral preparations can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass, plastic or other suitable material.

If administered intravenously, suitable carriers include physiological saline or phosphate buffered saline (PBS), and solutions containing thickening and solubilizing agents, such as glucose, polyethylene glycol, and polypropylene glycol and mixtures thereof. Liposomal suspensions, including tissue-targeted liposomes, may also be suitable as pharmaceutically acceptable carriers. These

may be prepared according to methods known to those skilled in the art. For example, lipsome formulations may be prepared as described in U.S. Pat. No. 4,522,811.

The active compounds may be prepared with carriers that protect the compound against rapid elimination from the body, such as time release formulations or coatings. Such carriers include controlled release formulations, such as, but not limited to, implants and microencapsulated delivery system, and biodegradable, biocompatible polymers, such as collagen, ethylene vinyl acetate, polyanhydrides, polyglycolic acid, polyorthoesters, polylactic acid and others. Methods for preparation of such formulations are known to those skilled in the art.

The compounds may be formulated for local or topical application, such as for topical application to the skin and mucous membranes, such as in the nose or eye, in the form of gels, creams, and lotions. The compounds may be formulated for application to the eye, or for intracisternal or intraspinal application in the form of solutions. Such solutions, particularly those intended for ophthalmic use, may be formulated as 0.01%-10% isotonic solutions, pH about 5-7, with appropriate salts. The compounds may be formulated as aerosols for topical application, such a by inhalation (see, e.g. U.S. Pat. Nos. 4,044,126, 4,414,209 and 4,364,923, which describe aerosols for delivery of a steroid useful for treating inflammatory diseases, particularly asthma).

Finally, the compounds may be packaged as kits containing packaging material, a compound identified by the method of the invention which is effective as an antineoplastic agent, ameliorating the symptoms of a neoplastic disorder, or activating the caspase cascade, and a packaging label that indicates that the compound or salt thereof is used as an antineoplastic agent, or for treating caspase cascade mediated disorders.

The following examples demonstrate usefulness of the invention in measuring the activity of caspases and other enzymes involved in apoptosis in cells and tissues. The examples also demonstrate usefulness of the invention in

drug screening assays that can be utilized to find enhancers of apoptosis. These examples are illustrative, but not limiting, of the method and compositions of the present invention. Other suitable modifications and adaptations of the variety of conditions and parameters normally encountered in *in vitro* assays and drug screening procedures or which are obvious to those skilled in the art are within the spirit and scope of the invention.

Example 1

Propidium iodide test for cell viability and membrane integrity

HL-60 cells were grown in Iscove's media (Life Technologies, Inc.) + 10% FCS (Sigma Chemical Company) in a 5% CO_2 -95% humidity incubator at 37 °C, and maintained at a cell density between 1 and 6 x 10^5 cells/ml. Cells were harvested at 600xg and resuspended at 2.2 x 10^6 cells/ml into Iscove's media + 1% FCS. An aliquot of 45 µl of cells was added to at least one well of a 96-well microtiter plate containing 5 µl of 10% DMSO in Iscove media solution containing the test compound as the test solution and at least one other well that is free of the test compound as a control sample. The samples were agitated and then incubated at 37 °C for 3 hr in a 5% CO_2-95% humidity incubator.

To each sample well there was added 50 µl of a solution of 10 µg/ml of propidium iodide in Iscove's media with 1% fetal calf serum. The samples were incubated for an additional 1 hour. Using a fluorescent plate reader (Model 1420, Wallac Instruments), an initial reading (T=0) was made approximately 1- 2 min after addition of the substrate solution, employing excitation at 520 nm and emission at 560 nm, to determine the background fluorescence of the control sample. After the 1-hr incubation at room temperature, the samples were read for fluorescence as above.

Calculation:

The Relative Fluorescence Unit values (RFU) were used to calculate the sample readings as follows:

$$RFU_{(T=1hr)} - RFU_{(T=0)} = \text{Net RFU}$$

The viability of the cell and integrity of the membrane was determined by the ratio of the net RFU value for test compound to that of control samples as follows:

$$\frac{\text{Net RFU of test compound sample}}{\text{Net RFU of control sample}} = \text{Ratio}$$

Viable cells with an integral cell membrane are those indicating a ratio of about 1 whereas non-viable cells with damaged membranes have ratios as high as 3.6.

Results are tabulated in Table 1 herein.

Example 2

Identification of vinblastine sulfate as an antineoplastic compound that exhibits caspase cascade activation

HL-60 cells were maintained and harvested as described in Example 1. An aliquot of 45 µl of HL-60 cells was added to a well of a 96-well microtiter plate containing 5 µl of a 10% DMSO in Iscove media solution containing 30 µg per ml of vinblastine sulfate. An aliquot of 45 µl of cells was added to a well of a 96-well microtiter plate containing 5 µl of a 10% DMSO in Iscove media solution without vinblastine as the control sample. The samples were mixed by agitation and then incubated at 37 °C for 3 hr in a 5% CO_2-95% humidity incubator. After incubation, the samples were removed from the incubator and 50 µl of a solution containing 20 µM of *N*-(Ac-DEVD)-*N'*-ethoxycarbonyl-R110 (SEQ ID NO:1) fluorogenic substrate, 20% sucrose (Sigma), 20 mM DTT (Sigma), 200 mM NaCl (Sigma), 40 mM Na PIPES buffer pH 7.2 (Sigma), and 500 µg/ml lysolecithin was added. The samples were mixed by agitation and incubated for 3 hr at room temperature. Using a fluorescent plate reader (Model

1420, Wallac Instruments), an initial reading (T=0) was made approximately 1-2 min after addition of the substrate solution, employing excitation at 485 nm and emission at 530 nm, to determine the background fluorescence of the control sample. After the 3 hr incubation, the samples were read for fluorescence as above (T = 3hr).

Calculation:

The Relative Fluorescence Unit values (RFU) were used to calculate the sample readings as follows:

$$\text{Sample RFU}_{(T=3hr)} - \text{Control RFU}_{(T=0)} = \text{Net RFU}_{(T=3hr)}$$

The potency of caspase cascade activation was determined by the ratio of the net RFU value for vinblastine to that of control samples as follows:

Ratio = 12.2

Thus, vinblastine is identified as an antineoplastic compound that is a caspase cascade activator in this assay.

Example 3

Identification of cyclophosphamide as an antineoplastic compound that does not exhibit caspase cascade activation

The assay was run under the same conditions described in Example 2 with vinblastine being replaced by cyclophosphamide.

The potency of caspase cascade activation was determined by the ratio of the net RFU value for cyclophosphamide to that of control samples as follows:

Ratio = 1.0

Thus, cyclophosphamide is identified as an antineoplastic compound that is not a caspase cascade activator in this assay.

Example 4

Multisample assay of known antineoplastic compounds for identifying compounds that exhibit caspase cascade activation (Table 1)

HL-60 cells were grown and harvested as in Example 1. Aliquots of 45 µl of cells, using an 8-channel multi-pipettor (Eppendorf), were added to wells of a 96-well microtiter plate -- each plate containing 5 µl of a 10% DMSO in Iscove media solution containing 30 µg per ml of a known antineoplastic, antibacterial, antimetabolite, immunosuppressive, cell poison or other compound. An aliquot of 45 µl of cells was added to a well of a 96-well microtiter plate containing 5 µl of a 10% DMSO in Iscove media solution without a test compound as the control sample. The samples were mixed by agitation and then incubated at 37 °C for 3 hr in a 5% CO_2-95% humidity incubator. After incubation, the samples were removed from the incubator and 50 µl of a solution containing 20 µM of *N*-(Ac-DEVD)-*N'*-ethoxycarbonyl-R110 (SEQ ID NO:1) fluorogenic substrate, 20% sucrose (Sigma), 20 mM DTT (Sigma), 200 mM NaCl (Sigma), 40 mM Na PIPES buffer pH 7.2 (Sigma), and 500 µg/ml lysolecithin was added using an 8-channel multi-pipettor (Eppendorf). The samples were mixed by agitation and incubated for 3 hr at room temperature. Using a fluorescent plate reader (Model 1420, Wallac Instruments), an initial reading (T=0) was made approximately 1- 2 min after addition of the substrate solution, employing excitation at 485 nm and emission at 530 nm, to determine the background fluorescence of the control sample. After the 3 hr incubation, the samples were read for fluorescence as above (T = 3hr).

Calculation for net RFU and ratio were carried out as described in Example 2. The potency of caspase cascade activation was determined by the ratio of the net RFU value for each sample to that of control samples to determine the potency of caspase cascade activation as shown in Table 1 herein. The assay detects therapeutically important known antineoplastic compounds which activate the caspase cascade. The assay also detects compounds which activate the

caspase cascade but are not known to be antineoplastic. The assay does not detect non-specific cell poisons or cytotoxic compounds.

Example 5

Multisample assay of a random library for identifying compounds that exhibit caspase cascade activation using 96-well liquid handling apparatus (Table 3)

HL-60 cells were grown and harvested as described in Example 1. All additions were made with a 96-well liquid handling apparatus (Quadra 96, Tomtec Instruments). Aliquots of 45 µl of cells was added to wells of a 96-well microtiter plate, each well containing 5 µl of a 10% DMSO in Iscove media solution containing 30 µg per ml of test compound. An aliquot of 45 µl of cells was added to a well of a 96-well microtiter plate containing 5 µl of a 10% DMSO in Iscove media solution without a test compound as the control sample. The samples were mixed by agitation and then incubated at 37 °C for 3 hr in a 5% CO_2-95% humidity incubator. After incubation, the samples were removed from the incubator and 50 µl of a solution containing 20 µM of *N*-(Ac-DEVD)-*N'*-ethoxycarbonyl-R110 (SEQ ID NO:1) fluorogenic substrate, 20% sucrose (Sigma), 20 mM DTT (Sigma), 200 mM NaCl (Sigma), 40 mM Na PIPES buffer pH 7.2 (Sigma), and 500 µg/ml lysolecithin was added. The samples were mixed by agitation and incubated for 3 hr at room temperature. Using a fluorescent plate reader (Model 1420, Wallac Instruments), an initial reading (T=0) was made approximately 1- 2 min after addition of the substrate solution, employing excitation at 485 nm and emission at 530 nm, to determine the background fluorescence of the control sample. After the 3 hr incubation, the samples were read for fluorescence as above (T = 3hr).

Calculations were carried out as described in Example 2. The potency of caspase cascade activation was determined by the ratio of the net RFU value for

each sample to that of control samples to determine the potency of caspase cascade activation as shown in Table 3 herein.

Example 6

Multisample assay of natural products for identifying compounds that exhibit caspase cascade activation using automated analysis equipment

The following procedure was conducted in an automated measurement apparatus for automatic drug screening. The apparatus is designed to initiate and measure events such as enzymatic transformations. The apparatus is designed to measure a resulting attribute of the sample for a period of time, record and process the resulting data, and present the processed data on a printer, magnetic storage device, or video display.

HL-60 cells were grown and harvested as described in Example 1. Aliquots of 45 µl of cells was added to wells of a 96-well microtiter plate, each of 80 wells containing 5 µl of a 10% DMSO in Iscove media solution containing 30 µg per ml of a natural product test compound. An aliquot of 45 µl of cells was added to a well of a 96-well microtiter plate containing 5 µl of a 10% DMSO in Iscove media solution without a test compound as the control sample. The samples were mixed by agitation and then incubated at 37 °C for 3 hr in a 5% CO_2-95% humidity incubator. After incubation, the samples were removed from the incubator and 50 µl of a solution containing 20 µM of *N*-(Ac-DEVD)-*N'*-ethoxycarbonyl-R110 (SEQ ID NO:1) fluorogenic substrate, 20% sucrose (Sigma), 20 mM DTT (Sigma), 200 mM NaCl (Sigma), 40 mM Na PIPES buffer pH 7.2 (Sigma), and 500 µg/ml lysolecithin was added. The samples were mixed by agitation and incubated for 3 hr at room temperature. Using a fluorescent plate reader (Victor Model 1420, Wallac Instruments, Bethesda, MD) an initial reading (T=0) was made approximately 1- 2 min after addition of the substrate solution, employing excitation at 485 nm and emission at 530 nm, to determine the

background fluorescence of the control sample. After the 3 hr incubation, the samples were read for fluorescence as above (T = 3hr).

Calculations were made as described in Example 2. The potency of caspase cascade activation was determined by the ratio of the net RFU value for each sample to that of control samples to determine the potency of caspase cascade activation as shown in Table 4 herein.

Example 7

Identification of antineoplastic compound that exhibits caspase cascade activation in solid tumor cells.

Solid tumor cells are frequently resistant to many standard chemotherapeutic agents and lack of activation of apoptosis indicates a lack of efficacy as an antineoplastic agent. To assess the ability to use solid tumor lines to identify antineoplastic compounds that exhibit caspase activation, T47D and ZR 75-1 human breast cancer lines or PC3 prostate cancer line were maintained with appropriate media (ATCC recommended media + 10% FCS). Cells were harvested and resuspended at $0.6x10^6$ cells/ml in media + 10% FCS. An aliquot of 45 µl of cells was added to each well of a 96-well microtiter plate. 5 µl of 100 µM vinblastine or 5 µl of 50 µM staurosporine or 5 µl of DMSO (solvent control) in RPMI media, was added to wells. The plates were incubated in a 5% CO_2-95% humidity incubator at 37 °C for 24 hr (T=24hr). After incubation, the samples were removed from the incubator and 50 µl of a solution containing 20 µM of *N*-(Ac-DEVD)-*N'*-ethoxycarbonyl-R110 (SEQ ID NO:1) fluorogenic substrate, 20% sucrose (Sigma), 20 mM DTT (Sigma), 200 mM NaCl (Sigma), 40 mM Na PIPES buffer pH 7.2 (Sigma), and 500 µg/ml lysolecithin (Calbiochem) was added. The samples were mixed by agitation and incubated for 3 hr at room temperature. Using a fluorescent plate reader (Model 1420, Wallac Instruments), an initial reading (T=0) was made approximately 1-2 min after addition of the substrate solution, employing excitation at 485 nm and emission at 530 nm, to

determine the background fluorescence of the control sample. After the 3 hr substrate incubation, the samples were read for fluorescence as above (T=24hr).

Calculation:

The Relative Fluorescence Unit values (RFU) were used to calculate the sample readings as follows:

$$RFU_{(T=24hr)} - \text{Control } RFU_{(T=0)} = \text{Net } RFU_{(T=24hr)}$$

The potency of caspase cascade activation was determined by the ratio of the net RFU value for the experimental compound to that of control samples as follows:

$$\text{Ratio} = \text{Net Sample } RFU_{(T=24hr)} / \text{Net Control } RFU_{(T=24hr)}$$

For these solid tumor cells vinblastine at 10 μM had a ratio of 2.1 with T47D cells, a ratio of 1.4 with ZR 75-1 cells, and a ratio of 1.3 with PC3 cells. The low ratios indicate that the compound is not effective with these resistant solid tumor cells. With staurosporine at 5 μM, the ratio was 6.7 with T47D, 9.5 with ZR 75-1 cells, and 2.5 with PC3 cells indicating a more potent caspase activation and more efficacy in cell killing.

Example 8

Identification of antineoplastic compound that exhibits caspase cascade activation without a permeabilizer for presenting reporter substrate to cells

Caspase activation can also be assessed when the reporter compound is presented directly to cells without a permeabilizer for the reporter substrate. T47D and ZR 75-1 human breast cancer lines or PC3 prostate cancer lines were maintained with appropriate media (ATCC recommended media + 10% FCS). Cells were harvested and resuspended at 0.6×10^6 cells/ml in media + 10% FCS. An aliquot of 45 μl of cells was added to each well of a 96-well microtiter plate. 5 μl of 100 μM vinblastine or 5 μl of 50 μM staurosporine or 5 μl of DMSO (solvent control) in RPMI media, was added to wells. The plates were incubated in a 5% CO_2-95% humidity incubator at 37 °C for 24 hr (T=24hr). After

incubation, the samples were removed from the incubator and 50 µl of a solution having concentrations of 20 µM of *N*-(Ac-DEVD)-*N'*-ethoxycarbonyl-R110 (SEQ ID NO:1) fluorogenic substrate, 20% sucrose (Sigma), 20 mM DTT (Sigma), 200 mM NaCl (Sigma), and 40 mM Na PIPES buffer pH 7.2 (Sigma) was added. The samples were mixed by agitation and incubated for 3 hr at room temperature. Using a fluorescent plate reader (Model 1420, Wallac Instruments), an initial reading (T=0) was made approximately 1-2 min after addition of the substrate solution, employing excitation at 485 nm and emission at 530 nm, to determine the background fluorescence of the control sample. After the 3 hr substrate incubation, the samples were read for fluorescence as above (T=24hr).

Calculations were made as described in Example 7. For these solid tumor cells vinblastine at 10 µM had a ratio of 5.2 with T47D cells, a ratio of 9.4 with ZR 75-1 cells, and a ratio of 12.7 with PC3 cells. With staurosporine at 5 µM, the ratio was 53.1 with T47D, 62.6 with ZR 75-1 cells, and 62.6 with PC3 cells. These ratios are higher than seen with a permeabilizer to present the reporter compound and the cut-off ratio for selection of antineoplastic agents can be increased to reflect these higher ratios. The lower ratios seen with vinblastine treatment indicate that the compound is not effective with these solid tumors compared to staurosporine. A quantitative method for determining an appropriate value for a minimum value for the ratio is to assess the control sample values and the standard deviation for the control sample: (Average Net RFU Control + 4x SD) / (Average Net RFU Control). This gives a statistically significant value to determine a cut-off value. Alternatively, in an evaluation with a large number of compounds, the mean and standard deviations of the inactive compounds can be used to determine an appropriate ratio. In these cases, the ratio for statistical significance (<0.01%) is greater than 7.5.

Example 9

Use of Pre-plated cells for the assay

Solid tumor cells grow and proliferate attached to a solid substratum. Examples 7 and 8 utilized solid tumor cells grown on a solid substratum then harvested and added directly to test sample. Assessed in this example was the effect on the level of caspase activation of harvesting cells, allowing time for adherence and then initiating treatment with test sample. T47D and ZR 75-1 human breast cancer lines or PC3 prostate cancer lines maintained with appropriate media (ATCC recommended media + 10% FCS) were used for evaluation. Cells grown attached to a solid substratum were harvested and resuspended at $0.6x10^6$ cells/ml in media + 10% FCS and 45 µl was added to wells of a 96-well plate and incubated overnight (16 hr) at 37 °C in a 5% CO2-95% humidity incubator to allow adherence to the assay plate (pre-plate). 5 µl of 100 µM vinblastine or 5 µl of 50 µM staurosporine or 5 µl of DMSO (solvent control) in RPMI media, was added to the wells and the samples incubated at 37 °C in a 5%CO2-95% humidity incubator for an additional 24 hr (T=24). Cells added at the time of compound addition were prepared and treated as described in Example 7. Caspase activity and the ratio of induction was measured for both conditions as described in Example 7. For these solid tumor cells, staurosporine at 5 µM showed with pre-plated cells a ratio of 7 with T47D cells, 18.3 with ZR75-1 cells, and 1.4 with PC3 cells. With compound addition at the same time as the cells, as in Example 7, the ratio was 7.6 for T47D cells, 15.3 for ZR75-1 cells, and 1.1 for PC3 cells, indicating little or no difference between the caspase induction levels, irrespective of whether cells were pre-plated or added at time of compound addition. Vinblastine at 10 µM showed marginal activation of caspase activity whether the cells were pre-plated or added at time of compound addition. These results demonstrate that cells either pre-plated or added at time of compound addition show little difference in the level of caspase activation.

On this basis, the instant invention should be recognized as constituting progress in science and the useful arts, as solving the problems in the identification of caspase cascade activators and potentially therapeutically effective antineoplastic agents.

Having described preferred embodiments of the invention, it is to be understood that the invention is not limited to those precise embodiments, and that the various changes and modifications may be effected therein by one skilled in the art without departing from the scope or spirit of the invention as defined in the appended claims. Thus, the scope of the invention should be determined by the appended claims and their legal equivalents, rather than by the examples given. All patents, patent applications and publications referred to herein are incorporated by reference in their entirety.

What Is Claimed Is:

1. A method for identifying a potentially therapeutically effective antineoplastic compound comprising determining the ability of a test compound to act as an activator of the caspase cascade in viable cultured eukaryotic cells having an intact cell membrane, wherein a test compound is determined to have potential therapeutic efficacy if said caspase cascade activity is enhanced in response to the presence of said test compound, said method comprising:

(a) obtaining viable cultured eukaryotic cells having an intact cell membrane expressing a cancer phenotype by culturing in a cell growth medium under conditions which result in growth;

(b) exposing said viable cultured cells to the test compound for a predetermined period of time at a predetermined temperature;

(c) adding a reporter compound having at least one measurable property which is responsive to the caspase cascade;

(d) measuring the caspase cascade activity of said exposed viable cultured cells by measuring said at least one measurable property; and,

(e) wherein an increase in the measured caspase cascade activity in the presence of said test compound is an indication that said test compound is a potential therapeutically effective antineoplastic compound.

2. The method of claim 1, wherein said cultured eukaryotic cells are higher eukaryotic cells.

3. The method of claim 1, wherein said cultured eukaryotic cells are mammalian cells.

4. The method of claim 1, wherein said cells are exposed to test compounds at concentrations in the range from about 1 picomolar to about 1 millimolar.

5. The method of claim 1, further comprising the addition of a permeabilization enhancer in combination with said reporter compound.

6. The method of claim 1, wherein said predetermined period of time is about 1 minute to about 24 hours; and, wherein said predetermined temperature is about 4 °C to about 50 °C.

7. The method of claim 6, wherein said predetermined period of time is about 1 hour to about 3 hours.

8. The method of claim 7, wherein said predetermined period of time is about 3 hours.

9. The method of claim 6, wherein said predetermined period of time is about 3 hours to about 24 hours.

10. The method of claim 9, wherein said predetermined period of time is about 5 hours.

11. The method of claim 9, wherein said predetermined period of time is about 24 hours.

12. The method of claim 1, wherein said reporter compound is selected from the group consisting of:

(a) a fluorogenic compound that produces fluorescence under the influence of the caspase cascade;

(b) a chromogenic compound that produces light absorption under the influence of the caspase cascade; and

(c) a chemiluminescent compound that produces light emission under the influence of the caspase cascade.

13. The method of claim 3, wherein said mammalian cells are a cell line selected from the group consisting of p53 deficient cells, Bcl-2 overexpressing family member cells, surviving overexpressing cells, Ataxia Telengiectasia Mutated cells, bcr/abl mutated cells, p16 mutated cells, Brca1 mutated cells, Brca 2 mutated cells, multi-drug resistance cells, and DNA mismatch repair deficiency cells.

14. The method of claim 1, with the proviso that a permeabilization enhancer is not added in combination with said reporter compound.

15. The method of claim 1, wherein a plurality of viable cultured cell samples are exposed separately to a plurality of test compounds.

16. The method of claim 15, wherein said plurality of viable cultured cells are in separate wells of a microtiter plate.

17. A method for assaying the potency of a potential therapeutically effective antineoplastic compound that functions as an activator of the caspase cascade in viable cultured eukaryotic cells having an intact cell membrane, said method comprising:

(a) obtaining a first and a second population of viable cultured eukaryotic cells having an intact cell membrane expressing a cancer phenotype by culturing said eukaryotic cells in a cell growth medium under conditions which result in growth;

(b) exposing said first population to a predetermined amount of a test compound dissolved in a solvent for a predetermined period of time at a predetermined temperature;

(c) exposing said second population to an amount of the solvent which was used to dissolve said test compound for said predetermined period of time at said predetermined temperature;

(d) adding to said test compound-exposed first population and said solvent-exposed second population a reporter compound having at least one measurable property which is responsive to the caspase cascade;

(e) measuring said at least one measurable property of said reporter compound in said test compound-exposed first population and thereby measuring the caspase cascade activity of said test compound-exposed first population;

(f) measuring said at least one measurable property of said reporter compound in said solvent-exposed second population and thereby measuring the caspase cascade activity of said solvent-exposed second population; and

(g) calculating the ratio of caspase cascade activity measured for said test compound-exposed first population compared to said caspase cascade activity measured for said solvent-exposed second population to determine the relative potency of the test compound as an activator of the caspase cascade.

18. The method of claim 17, wherein said cultured eukaryotic cells are higher eukaryotic cells.

19. The method of claim 17, wherein said cultured eukaryotic cells are mammalian cells.

20. The method of claim 17, wherein said cell lines are exposed to the test compound at a concentration in the range from about 1 picomolar to about 1 millimolar.

21. The method of claim 17, further comprising the addition of a permeabilization enhancer in combination with said reporter compound.

22. The method of claim 17, wherein said predetermined period of time is about 1 minute to about 24 hours; and, wherein said predetermined temperature is about 4 °C to about 50 °C.

23. The method of claim 22, wherein said predetermined period of time is about 1 hour to about 3 hours.

24. The method of claim 23, wherein said predetermined of time is about 3 hours.

25. The method of claim 22, wherein said predetermined period of time is about 3 hour to about 24 hours.

26. The method of claim 25, wherein said predetermined period of time is about 5 hours.

27. The method of claim 25, wherein said predetermined period of time is about 24 hours.

28. The method of claim 17, wherein said reporter compound is selected from the group consisting of:

(a) a fluorogenic compound that produces fluorescence under the influence of the caspase cascade;

(b) a chromogenic compound that produces light absorption under the influence of the caspase cascade; and,

(c) a chemiluminescent compound that produces light emission under the influence of the caspase cascade.

29. The method of claim 17, with the proviso that a permeabilization enhancer is not added in combination with said reporter compound.

30. The method of claim 17, wherein a plurality of first populations of viable cultured cells are exposed separately to a plurality of test compounds.

31. The method of claim 30, wherein said plurality of first populations of viable cultured cells are in separate wells of a microtiter plate.

32. A method for assaying the potency of a test compound to synergise with a known cancer chemotherapeutic agent by functioning as an activator of the caspase cascade, said method comprising:

(a) obtaining a first and a second population of viable cultured eukaryotic cells having an intact cell membrane expressing a cancer phenotype by culturing in a cell growth medium under conditions which result in growth;

(b) exposing said first population to a combination of a predetermined amount of a test compound and a subinducing amount of a known cancer chemotherapeutic agent for a first predetermined period of time, at a first predetermined temperature;

(c) exposing said second population to an amount of solvent which was used to dissolve the test compound and to a subinducing amount of a known cancer chemotherapeutic agent for said first predetermined period of time at said first predetermined temperature;

(d) adding a reporter compound to said exposed first population and to said exposed second population, said reporter compound having at least one measurable property which is responsive to the caspase cascade;

(e) incubating the resulting mixture of said first population, said test compound, said known cancer chemotherapeutic agent and said reporter

compound for a second predetermined time period at a second predetermined temperature;

(f) incubating the resulting mixture of said second population, said solvent, said known chemotherapeutic agent and said reporter compound for said second predetermined time period at said second predetermined temperature;

(g) measuring said at least one measurable property of said reporter compound in said first resulting mixture and thereby measuring the caspase cascade activity of said first population in said first resulting mixture;

(h) measuring said at least one measurable property of said reporter compound in said second resulting mixture and thereby measuring the caspase cascade activity of said second population in said second resulting mixture; and,

(i) calculating the ratio of measured caspase cascade activities of said first population to said second population to determine whether said test compound acts synergistically with said known cancer chemotherapeutic agent as an activator of the caspase cascade.

33. The method of any one of claims 17 or 32, wherein said mammalian cells are a cell line selected from the group consisting of p53 deficient cells, Bcl-2 overexpressing family member cells, survivin overexpressing cells, Ataxia Telengiectasia Mutated cells, bcr/abl mutated cells, p16 mutated cells, Brca1 mutated cells, Brca 2 mutated cells, multi-drug resistance cells, and DNA mismatch repair deficiency cells. .

34. The method of claim 32, wherein a plurality of first populations of viable cultured cells are exposed separately to a plurality of test compounds.

35. The method of claim 34, wherein said plurality of first populations of viable cultured cells are in separate wells of a microtiter plate.

36. A method of modulating *in vivo* apoptosis or neoplasia comprising administering to an animal in need of such modulation an effective amount of a compound, or a pharmaceutically acceptable salt or prodrug of said compound, which functions as a caspase cascade activator in the method of claim 1, with the proviso that said compound was first identified as a modulator of *in vivo* apoptosis or neoplasia in the method of claim 1.

37. A kit for performing the method of claim 36, comprising packaging material containing said compound, or a pharmaceutically acceptable salt or prodrug of said compound, said packaging material further comprising a label that indicates that said compound, or a pharmaceutically acceptable salt or prodrug of said compound, is useful to modulate *in vivo* apoptosis, or *in vivo* neoplastic disease.

38. A method of modulating *in vivo* apoptosis or neoplasia comprising administering to a subject in need of each modulation an effective amount of a compound, or a pharmaceutically acceptable salt or prodrug of said compound, which functions as a caspase cascade activator in the method of claim 17, wherein said ratio is at least about 2, with the proviso that said compound was first identified as a modulator of *in vivo* apoptosis or neoplasia in the method of claim 17.

39. The therapeutic method of claim 38, wherein said compound, or a pharmaceutically acceptable salt or prodrug of said compound, is selected from the group consisting of aklavin hydrochloride, citrinin, cytochalasin d, chloroquine, gramicidin, rotenone, cetylpyridinium chloride, emetine, digitoxin, and ouabain.

40. The therapeutic method of claim 38, wherein said compound, or a pharmaceutically acceptable salt of said compound, is selected from the group consisting of benzethonium chloride, baclofen, bithinolate sodium, apomorphine,

amphotericin B, colchicine, dehydrocholic acid, dienestrol, gentian violet, hexylresorcinol, disulfiram, gamma aminobutyric acid, furosemide, hydroxyprogesterone caproate, oxyphenbutazone, strophanthidin, tyrothricin, pimethixene, estradiol disulfate potassium salt, atrovenetin, gossypol-acetic acid, 7-chlorokynurenic acid, benzamil hydrochloride, amiodarone, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine hydrochloride, and verapamil hydrochloride.

41. The therapeutic method of claim 38, wherein said compound, or a pharmaceutically acceptable salt of said compound, is selected from the group consisting of 7-desacetoxy-6,7-dehydrogedunin, 6,2',3'-trihydroxy-7,4'-dimethoxyisoflavan, anthothecol, chukrasin, cedrelone, heteropeucenin methyl ether, gambogic acid, diacetyldiisovaleryl rhodomyrtoxin, sericetin diacetate, gamboginic acid methyl ester, gambogic acid amide, pomiferin, sumatrol acetate, brazilin, lonchocarpic acid diacetate, osajin, celastrol, orsellinic acid ethyl ester, gitoxigenin diacetate, strophanthidin, cymarin, stophanthidin acetate, periplocymarin, digoxin, emicymarin, strophanthidinic acid lactone acetate emetine, melatonin, chelidonine, norleagnine, calycanthine dictamine, dihydrogambogic acid, gambogic acid diethyl amide, crassin acetate, tetrahydrogambogic acid, decahydrogambogic acid, deoxodeoxydihydrogedunin, 1-(2-α-epoxy)-deacetoxydihydrogedunin, mexicanolide enol acetate, 4,4'-dimethoxydalberginone, methyl gamboginate, obtusaquinone, brazilin trimethyl ether, isorhodomyrtoxin, juglone, resistomycinolide, cucumin, dihydrocinchonidin, leodin diacetate, and 18α-glycyrrhetic acid.

42. A therapeutic method comprising administering to a subject an effective amount of a compound, or a pharmaceutically acceptable salt or prodrug of said compound, which functions as a caspase cascade activator in the method of claim 1, wherein said therapeutic method is useful to treat Hodgkin's disease, non-Hodgkin's lymphoma, acute or chronic lymphocytic leukemia, multiple

myeloma, neuroblastoma, breast carcinoma, ovarian carcinoma, lung carcinoma, Wilms' tumor, cervical carcinoma, testicular carcinoma, soft-tissue sarcoma, chronic lymphocytic leukemia, primary macroglobulinemia, bladder carcinoma, chronic granulocytic leukemia, primary brain carcinoma, small-cell lung carcinoma, stomach carcinoma, colon carcinoma, malignant pancreatic insulinoma, malignant carcinoid carcinoma, malignant melanoma, choriocarcinoma, mycosis fungoide, head or neck carcinoma, osteogenic sarcoma, pancreatic carcinoma, acute granulocytic leukemia, hairy cell leukemia, neuroblastoma, rhabdomyosarcoma, Kaposi's sarcoma, genitourinary carcinoma, thyroid carcinoma, esophageal carcinoma, malignant hypercalcemia, cervical carcinoma, renal cell carcinoma, endometrial carcinoma, polycythemia vera, essential thrombocytosis, adrenal cortex carcinoma, skin cancer, and prostatic carcinoma with the proviso that said compound was first identified in the method of claim 1 as therapeutically useful for such treatment.

43. A pharmaceutical composition effective to inhibit neoplasia comprising a first compound, or a pharmaceutically acceptable salt or prodrug of said compound, which functions as a caspase cascade activator, a second known cancer chemotherapeutic agent, and a pharmaceutically acceptable vehicle, with the proviso that said first compound was first identified as having potential antineoplastic activity in the method of claims 1, 17 or 32 and wherein said first and second compounds are present in amounts effective to inhibit neoplasia.

44. A pharmaceutical composition according to claim 43, wherein the measured potency of said first compound is at least about 2.

45. A pharmaceutical composition according to claim 43, wherein said first compound is selected from the group consisting of aklavin hydrochloride, citrinin, cytochalasin d, chloroquine, gramicidin, rotenone, cetylpyridinium chloride, emetine, digitoxin, and ouabain.

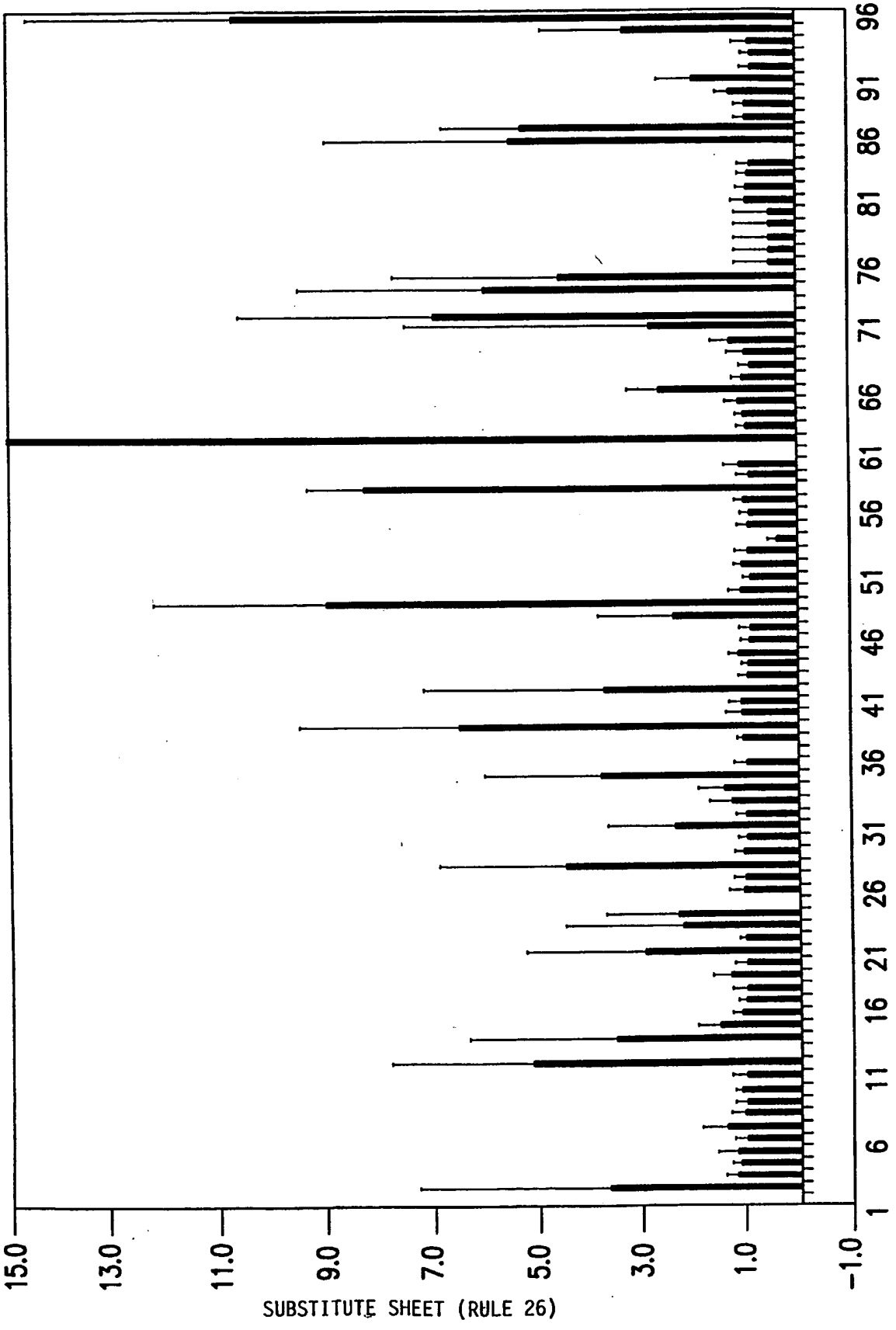
46. A pharmaceutical composition according to claim 43, wherein said first compound is selected from the group consisting of benzethonium chloride, baclofen, bithinolate sodium, apomorphine, amphotericin B, colchicine, dehydrocholic acid, dienestrol, gentian violet, hexylresorcinol, disulfiram, gamma aminobutyric acid, furosemide, hydroxyprogesterone caproate, oxyphenbutazone, strophanthidin, tyrothricin, pimethixene, estradiol disulfate potassium salt, atrovenetin, gossypol-acetic acid, 7-chlorokynurenic acid, benzamil hydrochloride, amiodarone, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine hydrochloride, and verapamil hydrochloride.

47. A pharmaceutical composition according to claim 43, wherein said first compound is selected from the group consisting of 7-desacetoxy-6,7-dehydrogedunin, 6,2',3'-trihydroxy-7,4'-dimethoxyisoflavan, anthothecol, chukrasin, cedrelone, heteropeucenin methyl ether, gambogic acid, diacetyldi-isovaleryl-rhodomyrtoxin, sericetin diacetate, gamboginic acid methyl ester, gambogic acid amide, pomiferin, sumatrol acetate, brazilin, lonchocarpic acid diacetate, osajin, celastrol, orsellinic acid ethyl ester, gitoxigenin diacetate, strophanthidin, cymarin, stophanthidin acetate, periplocymarin, digoxin, emicymarin, strophanthidinic acid lactone acetate, emetine, melatonin, chelidonine, norleagnine, calycanthine dictamine, dihydrogambogic acid, gambogic acid diethyl amide, crassin acetate, tetrahydrogambogic acid, decahydrogambogic acid, deoxodeoxydihydrogedunin, 1-(2-α-epoxy)-deacetoxydihydrogedunin, mexicanolide enol acetate, 4,4'-dimethoxydalberginone, methyl gamboginate, obtusaquinone, brazilin trimethyl ether, isorhodomyrtoxin, juglone, resistomycinolide, cucumin, dihydrocinchonidin, leodin diacetate, and 18α-glycyrrhetic acid.

48. A composition according to claim 43, wherein said inhibition achieved by a predetermined amount of said composition is greater than the

additive effect achieved by using said first compound and said second compound individually in the amounts that each comprises in said predetermined amount of said composition.

FIG. 1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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/02329

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) : G01N 33/48; C12Q 1/00
US CL : 436/63; 435/4
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 436/63; 435/4

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EAST, STN

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MOHR et al. Marcophages resistant to endogenously generated nitic oxide-mediated apoptosis are hypersensitive to exogenously added nitric oxide donors:Dichotomous apoptotic response independent of caspase 3 and reversal by the mitogen-activated protein kinase kinase (MEK) inhibitor PD 098059, Proc. Natl. Acad. Sci. USA. April 1998, Vol. 95, pages 5045-5050, especially page 5045.	1-48
X	FULDA et al. Betulinic acid triggers CD95 (APO-1/Fas)- and p53-independent apoptosis via activation of caspases in neuroectodermal tumors. Cancer Research. 01 November 1997, Vol. 57, pages 4656-4964, especially page 4656.	1-48

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
27 MARCH 2000	10 MAY 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer Dorethea Laurence for JENNIFER E. NICHOLS, NEE HUNT
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/02329

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	QI et al. Baculovirus p35 and Z-VAD-fmk inhibit thapsigargin-induced apoptosis of breast cancer cells. Oncogene. 1997, Vol. 15, pages 1207-1212, especially page 1212.	1-48